PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12Q 1/02, 1/00, G01N 33/567, 33/574, 33/53, C12P 21/06, A61K 38/00, 38/04, 35/14, A01N 37/18

A1

(11) International Publication Number: **WO 98/22617**

(43) International Publication Date: 28 May 1998 (28.05.98)

(21) International Application Number: PCT/US97/21674

(22) International Filing Date: 20 November 1997 (20.11.97)

(30) Priority Data:

08/754,322	21 November 1996 (21.11.96)	US
08/915,189	20 August 1997 (20.08.97)	US
Not furnished	18 November 1997 (18.11.97)	US

(71) Applicant *(for all designated States except US)*: THE REGENTS OF THE UNIVERSITY OF MICHIGAN [US/US]; Wolverine Tower, Room 2071, 3003 South State Street, Ann Arbor, MI 48109 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: LIVANT, Donna, L. [US/US]; 2625 Antietam Drive, Ann Arbor, MI 48105 (US).

(74) Agents: CARROLL, Peter, G. et al.; Medlen & Carroll, LLP, Suite 2200, 220 Montgomery Street, San Francisco, CA 94104 (US).

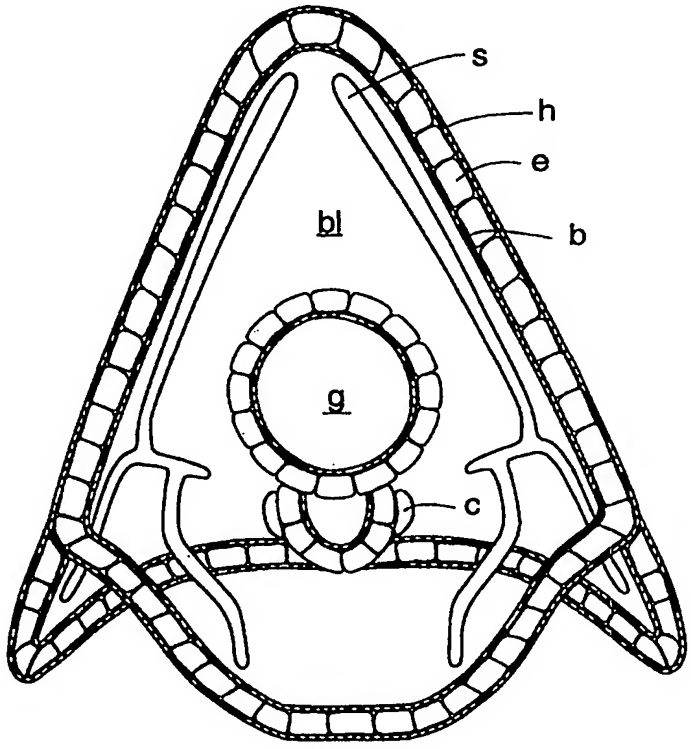
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: INVASION–INDUCING AGENTS AND INVASION–INHIBITORS FOR USE IN WOUND HEALING AND CANCER

(57) Abstract

Wound healing–promoting and cancer–inhibiting compounds are described. Specifically, therapeutic agents are disclosed which a) promote wound healing, or b) exhibit anti–metastatic and anti–growth properties. In addition, screening assays are provided for identifying additional therapeutic agents. The figure schematically shows the embodiment of the substrate used according to the present invention for testing tumor cells.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INVASION-INDUCING AGENTS AND INVASION-INHIBITORS FOR USE IN WOUND HEALING AND CANCER

This application is a continuation-in-part of U.S. Patent applications Ser. Nos. 08/915,189 (filed August 20, 1997) and _____ (filed November 18, 1997), both which are continuations-in-part of U.S. Patent Application Ser. No. 08/754,322 filed on November 21, 1996.

FIELD OF THE INVENTION

The present invention relates to wound healing and the treatment of cancer as well as to the identification and use of drugs to promote wound healing or inhibit tumor invasion and growth.

BACKGROUND

The term "chemotherapy" simply means the treatment of disease with chemical substances. The father of chemotherapy, Paul Ehrlich, imagined the perfect chemotherapeutic as a "magic bullet"; such a compound would kill invading organisms without harming the host. This target specificity is sought in all types of chemotherapeutics, including anticancer agents.

However, specificity has been the major problem with anticancer agents. In the case of anticancer agents, the drug needs to distinguish between host cells that are cancerous and host cells that are not cancerous. The vast bulk of anticancer drugs are indiscriminate at this level. Typically anticancer agents have negative hematological effects (*e.g.*, cessation of mitosis and disintegration of formed elements in marrow and lymphoid tissues), and immunosuppressive action (*e.g.*, depressed cell counts), as well as a severe impact on epithelial tissues (*e.g.*, intestinal mucosa), reproductive tissues (*e.g.*, impairment of spermatogenesis), and the nervous system. P. Calabresi and B.A. Chabner, In: Goodman and Gilman, The Pharmacological Basis of Therapeutics (Pergamon Press, 8th Edition) (pp. 1209-1216).

Success with chemotherapeutics as anticancer agents has also been hampered by the phenomenon of multiple drug resistance, resistance to a wide range of structurally unrelated cytotoxic anticancer compounds. J.H. Gerlach *et al.*, *Cancer Surveys*, 5:25-46 (1986). The underlying cause of progressive drug resistance may be due to a small population of drug-

resistant cells within the tumor (*e.g.*, mutant cells) at the time of diagnosis. J.H. Goldie and Andrew J. Coldman. *Cancer Research*, 44:3643-3653 (1984). Treating such a tumor with a single drug first results in a remission, where the tumor shrinks in size as a result of the killing of the predominant drug-sensitive cells. With the drug-sensitive cells gone, the remaining drug-resistant cells continue to multiply and eventually dominate the cell population of the tumor.

Finally, the treatment of cancer has been hampered by the fact that there is considerable heterogeneity even within one type of cancer. Some cancers, for example, have the ability to invade tissues and display an aggressive course of growth characterized by metastases. These tumors generally are associated with a poor outcome for the patient. And yet, without a means of identifying such tumors and distinguishing such tumors from non-invasive cancer, the physician is at a loss to change and/or optimize therapy.

What is needed is a specific anticancer approach that is reliable for a wide variety of tumor types, and particularly suitable for invasive tumors. Importantly, the treatment must be effective with minimal host toxicity.

SUMMARY OF THE INVENTION

The present invention relates to wound healing and the treatment of cancer, to the testing of cancer cells for their ability to invade tissues and cause metastases, and to the identification and use of drugs to a) promote wound healing, and b) inhibit tumor invasion and growth. In addition, the present invention provides compounds useful as anti-coagulants, adjuvants, and enhancers in gene therapy.

In one embodiment, the present invention provides: A) an *in vitro* model for testing cancer cells and evaluating invasive potential; B) a screening assay for identifying drugs that inhibit tumor invasion; and C) chemotherapeutics for treating cancer. A variety of assay formats are contemplated for testing the invasive potential of cancer cells. In one embodiment, a portion of a patient's tumor is obtained (*e.g.*, by biopsy) and placed in tissue culture on a fibronectin-free substrate. Thereafter, the response of the tumor cells to fibronectin or a fibronectin-derived peptide is assessed. Where fibronectin induces invasion of the membrane, the tumor can be considered to have metastatic potential. Where there is no significant invasion of the membrane, the tumor can be considered (at that time) to be non-metastatic.

In one embodiment, the present invention contemplates a method of evaluating human cancer comprising: a) providing: i) a human cancer patient, ii) a fibronectin-free substrate, and iii) one or more invasion-inducing agents; b) obtaining cancer cells from said patient; c) contacting said cells *ex vivo* with said fibronectin-free substrate and one or more invasion-inducing agents; and d) detecting cancer cell invasion of said substrate. Preferably the cancer cells are cultured (or at least briefly maintained) in serum-free culture media so as to essentially avoid introducing complicating serum factors. In one embodiment, the invasion-inducing agent is a peptide, said peptide comprising the sequence PHSRN (Seq. ID No. 1). In one embodiment, the invasion inducing agent is a plasma fibronectin-derived peptide.

While not limited to any mechanism, it is believed that cells exposed to invasion-inducing agents in this manner are potentially rendered capable of invading the substrate. Indeed, the present invention contemplates stimulation of invasion by all cells of the body, including, but not limited to: epithelial cells (keratinocytes, mammary and prostate epithelial cells), connective tissue (fibroblasts), and muscle (myoblast) cells. Again, while not limited to any mechanism, it is believed that the invasion inducing agent comprising the fibronectin sequence PHSRN binds to the $\alpha 5\beta 1$ receptor on the cancer cell and thereby induces invasion of the substrate. In this regard, the present invention provides a method of testing human cancer cells comprising: a) providing: i) a human cancer patient, ii) a fibronectin-free substrate, and iii) one or more invasion-inducing agents; b) obtaining $\alpha 5\beta 1$ integrin fibronectin receptor-expressing cancer cells from said patient; c) culturing (or maintaining) said cells in serum-free culture media on said substrate in the presence of said invasion-inducing agents; and d) detecting cancer cell invasion of said substrate.

As noted above, the present invention also contemplates a screening assay for identifying drugs that inhibit tumor invasion. The present invention contemplates a screening assay utilizing the binding activity of fibronectin-derived peptides. In one embodiment, an inducible tumor cell line is placed in tissue culture on a fibronectin-free substrate. Thereafter, as an inducible tumor cell line, the tumor will be induced (under ordinary conditions) by fibronectin or the fibronectin-derived peptide to invade the substrate. However, in this drug screening assay, candidate drug inhibitors are added to the tissue culture (this can be done individually or in mixtures). Where the inducible tumor cell is found to be inhibited from invading the substrate, a drug inhibitor is indicated.

It is not intended that the present invention be limited by the nature of the drugs screened in the screening assay of the present invention. A variety of drugs, including peptides, are contemplated.

Finally, the present invention contemplates chemotherapeutics for treating invasive tumors. Specifically, a variety of anti-invasive chemotherapeutic agents are contemplated to antagonize the invasion-promoting activity of the PHSRN peptide. In the preferred embodiment, the invasion-inhibitor or "anti-invasive agent" is a peptide with the amino acid sequence PHSCN (Seq. ID No. 2) (and corresponding peptide and non-peptide mimetics). In another embodiment, the anti-invasive agent is a peptide which has an amino acid sequence comprising a sequence selected from the group consisting of CHSRN (Seq. ID No. 3), PCSRN (Seq. ID No. 4), PHCRN (Seq. ID No. 5), and PHSRC (Seq. ID No. 6). In another embodiment, the anti-invasive agent is an invasion-inhibiting peptide which has an amino acid sequence comprising PHSXN, where X is an amino acid selected from the group consisting of homo-cysteine, the D-isomer of cysteine, histidine, and penicillamine.

The present invention also contemplates an anti-invasive agent (*e.g.* invasion-inhibiting peptide) comprising the amino acid sequence X_1HSX_2N, wherein X_1 is either proline, histidine, an amino acid analogue or not an amino acid, and X_2 is an amino acid selected from the group consisting of the L-isomer of cysteine, the D-isomer of cysteine, homo-cysteine, histidine, and penicillamine. In another embodiment, the present invention contemplates an anti-invasive agent comprising the amino acid sequence $X_1X_2X_3X_4X_5$, wherein X_1 is an amino acid selected from the group consisting of proline, glycine, valine, histidine, isoleucine, phenylalanine, tyrosine, and tryptophan, and X_2 is an amino acid selected from the group consisting of histidine, proline, tyrosine, glycine, asparagine, glutamine, arginine, lysine, phenylalanine, and tryptophan, and X_3 is an amino acid selected from the group consisting of serine, threonine, alanine, tyrosine, leucine, histidine, asparagine, glycine and glutamine, and X_4 is an amino acid selected from the group consisting of cysteine, homo-cysteine, penicillamine, histidine, tyrosine, asparagine, glutamine, and methionine, and X_5 is an amino acid selected from the group consisting of asparagine, glutamine, serine, threonine, histidine, glycine and tyrosine. In the preferred embodiment the peptide is PHSCN, where the cysteine is either the L-isomer or D-isomer.

It is further contemplated that the anti-invasive agents named above comprise the named amino acid sequence and additional amino acids added to the amino terminus, the carboxyl terminus, or both the amino and carboxyl termini. In one embodiment, the anti-

invasive agent is up to five hundred amino acids in length, and more preferably between four and five hundred amino acids, and still more preferably between six and one hundred amino acids. It is also contemplated that, in some embodiments, the anti-invasive agents named above comprise a peptide with the amino terminus blocked by standard methods to prevent digestion by exopeptidases, for example by acetylation; and the carboxyl terminus blocked by standard methods to prevent digestion by exopeptidases, for example, by amidation. Further, it is contemplated that, in some embodiments, the anti-invasive agents named above comprise a peptide having one or more L-amino acids replaced by their D-isomers to prevent digestion by endoproteinases.

In this regard, the present invention provides a method of treating cancer comprising: a) providing: i) a subject having cancer, and ii) a composition of matter comprising a peptide which inhibits the tumor invasion-promoting activity of the PHSRN sequence of plasma fibronectin; and b) administering said composition to said subject. The present invention further contemplates using antagonists before and/or after surgical removal of the primary tumor. In one embodiment, the method comprises administering a PHSRN antagonist as adjunct therapy with additional chemotherapeutics.

While not limited to any mechanism, it is believed that these anti-invasive chemotherapeutic agents antagonize the invasion-promoting activity of the PHSRN sequence (*e.g.*, of fibronectin) by blocking the binding of this sequence to its receptor on tumor cells. Again, while not limited to any mechanism, it is believed that the PHSRN sequence may promote invasion by acting to displace a divalent cation (Mg+2, Ca+2, or Mn+) in the $\alpha 5\beta 1$ receptor on metastatic tumor cells, and the above named chemotherapeutic anti-invasive agents might act to inhibit this invasion by chelating one or more of these divalent cations.

In another embodiment, the present invention contemplates anti-invasion antagonists to the IKVAV sequence of laminin, including but not limited to, peptides comprising the structure, ICVAV, and corresponding peptide mimetics.

The present invention also relates to methods and compositions for wound healing, and in particular, methods and compositions to promote and enhance wound healing, especially chronic wounds (*e.g.*, diabetic wounds, pressure sores). The compositions of the present invention are based on the discovery that peptides containing the amino acid sequence PHSRN promote wound healing. The present invention contemplates the use of such peptides, peptide derivatives, protease-resistant peptides, and non-peptide mimetics in the treatment of wounds.

It is not intended that the present invention be limited to the mode by which the compositions of the present invention are introduced to the patient. In one embodiment, the present invention contemplates systemic administration of the compound (e.g. intravenous). In another embodiment, the present invention contemplates topical administration, including but not limited to topical administration using solid supports (such as dressings and other matrices) and medicinal formulations (such as mixtures, suspensions and ointments). In one embodiment, the solid support comprises a biocompatible membrane. In another embodiment, the solid support comprises a wound dressing. In still another embodiment, the solid support comprises a band-aid.

The present invention contemplates a method for treating a wound, comprising a) providing: i) an invasion-inducing agent, and ii) a subject having at least one wound; and b) administering said invasion-inducing agent to said subject under conditions such that the healing of said wound is promoted.

The present invention also contemplates a method for treating a wound, comprising a) providing: i) an invasion-inducing agent on a solid support, and ii) a subject having at least one wound; and b) placing the solid support into the wound of the subject under conditions such that the healing of the wound is promoted.

The present invention also contemplates a method of screening candidate invasion-inducing agents useful in wound healing comprising: a) providing: i) inducible cells, ii) a fibronectin-depleted substrate, and iii) one or more candidate invasion-inducing agents, b) contacting said cells *in vitro* with said fibronectin-free substrate and said one or more candidate invasion-inducing agents; and c) measuring the extent of cell invasion of said substrate. It is not intended that the present invention be limited to the type of inducible cells. In one embodiment, said inducible cells are epithelial cells. In another embodiment, said inducible cells are selected from the group consisting of fibroblasts, keratinocytes and muscle cells.

It is also not intended that the present invention be limited to a particular invasion-inducing agent. In one embodiment, the invasion -inducing agent is a peptide comprising the amino acid sequence X_1HSX_2N, wherein X_1 is either proline, histidine, an amino acid analogue or not an amino acid, and X_2 is an amino acid selected from the group consisting of the L-isomer of arginine and the D-isomer of arginine. In one embodiment, said invasion-inducing agent comprises a fibronectin-derived peptide. In one embodiment, the present invention contemplates a fibronectin-derived peptide comprising amino acids, wherein at least

a portion of said peptide comprises the sequence PHSRN. In a preferred embodiment, said peptide comprises the amino acid sequence PHSRN. In yet another embodiment, said peptide lacks the RGD motif. In yet another embodiment, said peptide lacks the motif which binds the $\alpha 5\beta 1$ receptor.

It is not intended that the present invention be limited by the length of the peptide. In one embodiment, said peptide is between five and five hundred amino acids in length. In a preferred embodiment, said peptide comprises the amino acids PHSRN and additional amino acids added to the amino terminus. In another embodiment, said peptide comprises the amino acids PHSRN and additional amino acids added to the carboxy terminus. In yet another embodiment, said peptides comprises the amino acids PHSRN and additional amino acids added to both the amino and carboxy termini.

It is not intended that the present invention be limited to specific invasion-inducing agents. In one embodiment, the present invention contemplates invasion-inducing agents that comprise peptides that are protease resistant. In one embodiment, such protease-resistant peptides are peptides comprising protecting groups. In a preferred embodiment, endoprotease-resistance is achieved using peptides which comprise at least one D-amino acid.

The present invention also contemplates using fibronectin-derived peptides (as well as derivatives and non-peptide mimetics) as anti-coagulants, adjuvants, and enhancers in gene therapy. In particular, the present invention contemplates using fibronectin-derived peptides as anti-coagulants, adjuvants, and enhancers in gene therapy, wherein said peptides lack the motif which binds the $\alpha 5\beta 1$ receptor.

With regard to anti-coagulants, the present invention contemplates introducing the fibronectin-derived peptides (as well as derivatives and non-peptide mimetics) of the present invention into blood, serum or plasma. In one embodiment, the present invention contemplates introducing a PHSCN-containing peptide (including but not limited to such a peptide which has been chemically modified to render it more resistant to proteases) into blood, such as by intravenous administration to a subject in need of an anti-coagulant.

With regard to adjuvants, the present invention contemplates using a mixture of antigen and the fibronectin-derived peptides (as well as derivatives and non-peptide mimetics) of the present invention to immunize animals, including humans. In one embodiment, the present invention contemplates mixing antigen with a PHSRN-containing peptide (including but not limited to such a peptide which has been chemically modified to render it more

resistant to proteases) and immunizing (whether subcutaneously, intramuscularly, intraperatoneally, etc.) with the mixture to generate antibody to the antigen.

Thus, the present invention specifically contemplates compositions (including but not limited to vaccines) comprising antigen (such as antigens from pathogens, said pathogens including but not limited to bacterial and viral pathogens) and the fibronectin-derived peptides (such as a PHSRN-containing peptide) of the present invention. In one embodiment, the present invention contemplates using the compositions of the present invention as adjuvants where the animal to be immunized displays lower immune responses to antigen than controls. For example, in the case of humans who are partially immunocompromised (such as humans exposed to the AIDS virus), the compositions of the present invention may improve the response to the immunogen (such as the response to the antigens of the AIDS virus).

With regard to gene therapy, the present invention contemplates using a mixture comprising nucleic acid (whether DNA or RNA, or nucleic acid containing nucleotide analogues) and the fibronectin-derived peptides (as well as derivatives and non-peptide mimetics) of the present invention to introduce nucleic acid into the cells of animals, including humans. In one embodiment, the present invention contemplates mixing nucleic acid encoding a gene of interest (and preferably a gene encoding a protein having beneficial properties to the receiving host) with a PHSRN-containing peptide (including but not limited to such a peptide which has been chemically modified to render it more resistant to proteases) and transfecting cells (whether those cells are liver cells, muscle cells, nerve cells, etc.) with the mixture (whether *in vivo* or *in vitro*) to introduce said nucleic acid into said cells. In one embodiment, the nucleic acid is contained within a vector and the nucleic acid is introduced under conditions such that said gene of interest is expressed. Thus, the present invention specifically contemplates compositions comprising nucleic acid (such as nucleic acid encoding a protein of interest, said protein of interest including but not limited to proteins having a beneficial impact) and the fibronectin-derived peptides (such as a PHSRN-containing peptide) of the present invention.

DESCRIPTION OF THE FIGURES

Figure 1 schematically shows the one embodiment of the substrate used according to the present invention for testing tumor cells. The spatial relationship of the ectoderm of the *Strongylocentrotus purpuratus* embryo to its extracellular matrix and to blastocoelar structures are shown (s, spicules; h, hyalin layer; e, ectoderm; b, subectodermal basement membrane; bl,

blastocoel; g, stomach of the primitive gut; c, coelomic pouches). The esophagus and intestine do not appear on the side of the embryo shown.

Figure 2 is a graph showing the results of the testing of tumor cells on fibronectin-containing substrates and fibronectin-depleted substrates *in vitro* without the use of the invasion-inducing agents of the present invention.

Figure 3 is a graph showing the results of the testing of tumor cells on fibronectin-depleted substrates *in vitro* with and without invasion-inducing agents according one embodiment of the method of the present invention.

Figure 4 is a graph showing the results of the testing of normal cells on fibronectin-depleted substrates *in vitro* with and without invasion-inducing agents according one embodiment of the method of the present invention.

Figure 5A is a graph showing the results of inhibiting serum-induced human breast cancer cell invasion of the SU-ECM substrate with varying concentrations of the PHSCN peptide.

Figure 5B is a graph showing the results of inhibiting PHSRN-induced invasion by both human breast cancer cells and normal human mammary epithelial cells of the SU-ECM substrate with varying concentrations of the PHSCN peptide.

Figure 6A is a graph showing the results of inhibiting serum-induced human prostate cancer cell invasion of the SU-ECM substrate with varying concentrations of the PHSCN peptide.

Figure 6B is a graph showing the results of inhibiting PHSRN-induced invasion by both human prostate cancer cells and normal prostate epithelial cells of the SU-ECM substrate with varying concentrations of the PHSCN peptide.

Figure 7A is a graph showing the results of testing serum-induced rat prostate cancer cell invasion of the SU-ECM substrate with and without the PHSCN peptide.

Figure 7B is a graph showing the results of inhibiting PHSRN-induced rat prostate cancer cell invasion of the SU-ECM substrate with varying concentrations of the PHSCN peptide.

Figure 8 is a graph showing the results of inhibiting serum-induced rat prostate cancer cell invasion of the SU-ECM substrate with varying concentrations of the PHS(homo)CN peptide.

Figure 9A is a graph showing the results of testing tumor growth in rats injected with prostate cancer cells, with half of the rats receiving treatment with the PHSCN peptide, initiated in conjunction with the initial injection.

Figure 9B is a graph showing the results of determining the mean number of lung metastases in the two groups of rats described in Figure 9a.

Figure 10A is a graph showing the results of testing tumor growth in rats injected with prostate cancer cells, with half of the rats receiving treatment with the PHSCN peptide, initiated 24 hours after the initial cancer cell injection.

Figure 10B is a graph showing the results of determining the mean number of lung metastases in the two groups of rats described in Figure 10a.

Figure 10C is a graph showing the results of determining the mean mass of intraperitoneal metastatic tissues in the two groups of rats described in Figure 10a.

Figure 11 is a graph showing the results of inhibiting serum-induced human cancer cell invasion with varying concentrations of the PHSCN peptide, as well as PHSCN peptide that has been chemically modified with protecting groups and PHSCN peptide wherein an L-amino acid has been replaced with the D-isomer.

Figure 12 is a graph showing the results of inhibiting serum-induced human cancer cell invasion with varying concentrations of non-peptide compounds in comparison to the PHSCN peptide, as well as PHSCN peptide that has been chemically modified with protecting groups and PHSCN peptide wherein an L-amino acid has been replaced with the D-isomer.

Figure 13 is a schematic showing structural relationships between PHSCN and the non-peptide compounds of Figure 12.

Figure 14 is a graph showing the results of attempts to inhibit serum-induced human cancer cell invasion with varying concentrations of bis(diethylthiocarbamoyl) disulfide ("Antabuse").

Figure 15 is a graph showing the percentages of invaded neonatal fibroblasts, corresponding to various fragments of the plasma fibronectin cell binding domain, after placement on an invasion substrates. The 120 kDa and 39 kDa fragments contain the PHSRN sequence. The 11.5 kDa fragment does not. These fragments lack the $\alpha 4\beta 1$ integrin binding site in the IIICS region.

Figure 16 is a graph presenting a dose response curve relating concentration of peptides containing the amino acid sequence PHSRN to fibroblast invasion into an invasion substrate.

Figure 17 is a graph presenting a dose response curve relating concentration of peptides containing the amino acid sequence PHSRN to keratinocyte invasion into an invasion substrate.

Figure 18 is a graph presenting a dose response curve relating concentration of peptides containing the amino acid sequence PHSRN to human mammary or prostate epithelial cell invasion into an invasion substrate.

Figure 19 is a graph presenting the inductive effect of peptides containing the amino acid sequence PHSRN on mouse muscle satellite cell invasion into an invasion substrate.

Figure 20 is a graph presenting dermal wound closure data as a function of time in genetically obese / diabetic mice, and their controls, in response to treatment with peptides containing the amino acid sequence PHSRN.

Figure 21 is a graph presenting dermal wound closure data as a function of time in diabetic and non-diabetic mice treated with peptides containing the amino acid sequence PHSRN.

Figure 22 is a graph presenting the percentages of closed wounds as a function of time in diabetic mice treated with peptides containing the amino acid sequence PHSRN.

Figure 23 is a graph showing the inhibition of the PHSCN peptide on blood clotting.

DEFINITIONS

The term "drug" as used herein, refers to any medicinal substance used in humans or other animals. Encompassed within this definition are compound analogs, naturally occurring, synthetic and recombinant pharmaceuticals, hormones, antimicrobials, neurotransmitters, etc.

The term "inducing agent" refers to any compound or molecule which is capable of causing (directly or indirectly) the invasion of cells in a substrate. Thus, invasion inducing agents are defined functionally. This function can be readily assessed by using the invasion substrates and assays of the present invention (described below). "Inducing agents" include, but are not limited to, PHSRN-containing peptides and related peptides (see below).

The term "receptors" refers to structures expressed by cells and which recognize binding molecules (*e.g.*, ligands).

The term "antagonist" refers to molecules or compounds which inhibit the action of a "native" or "natural" compound (such as fibronectin). Antagonists may or may not be homologous to these natural compounds in respect to conformation, charge or other characteristics. Thus, antagonists may be recognized by the same or different receptors that

are recognized by the natural compound. "Antagonists" include, but are not limited to, PHSCN-containing peptides and related peptides (see below).

The term "host cell" or "cell" refers to any cell which is used in any of the screening assays of the present invention. "Host cell" or "cell" also refers to any cell which either naturally expresses particular receptors of interest or is genetically altered so as to produce these normal or mutated receptors. Cells can be transfected with nucleic acid encoding a gene of interest (*i.e.* a gene encoding a particular protein, including but not limited to proteins that are therapeutic). The present invention contemplates that the peptides (and derivatives and mimetics) of the present invention are useful to facilitate and enhance the process of introducing nucleic acid into cells.

The term "chemotherapeutic agent" refers to molecules or compounds which inhibit the growth or metastasis of tumors. "Chemotherapeutics" include, but are not limited to, PHSCN-containing peptides and related peptides (see below).

The present invention also contemplates homo-cysteine, which is identified as "hC".

The term "wound" refers broadly to injuries to the skin and subcutaneous tissue initiated in different ways (*e.g.*, pressure sores from extended bed rest and wounds induced by trauma) and with varying characteristics. Wounds may be classified into one of four grades depending on the depth of the wound: i) Grade I: wounds limited to the epithelium; ii) Grade II: wounds extending into the dermis; iii) Grade III: wounds extending into the subcutaneous tissue; and iv) Grade IV (or full-thickness wounds): wounds wherein bones are exposed (*e.g.*, a bony pressure point such as the greater trochanter or the sacrum). The term "partial thickness wound" refers to wounds that encompass Grades I-III; examples of partial thickness wounds include burn wounds, pressure sores, venous stasis ulcers, and diabetic ulcers. The term "deep wound" is meant to include both Grade III and Grade IV wounds. The present invention contemplates treating all wound types, including deep wounds and chronic wounds.

The term "chronic wound" refers to a wound that has not healed within 30 days.

The phrase "positioning the solid support in or on the wound" is intended to mean contacting some part of the wound with the solid support.

The phrases "promote wound healing," "enhance wound healing," and the like refer to either the induction of the formation of granulation tissue of wound contraction and/or the induction of epithelialization (*i.e.*, the generation of new cells in the epithelium). Wound healing is conveniently measured by decreasing wound area.

The phrase "wound fluid contents" refers to liquid associated with a wound, as well as cells, cell factors, ions, macromolecules and protein material suspended such liquid at the wound site.

The term "subject" refers to both humans and animals.

The terms "enclosure," "compartment," and the like refer broadly to any container capable of confining a solid support within a defined location.

The term "solid support" refers broadly to any support, including, but not limited to, microcarrier beads, gels, Band-Aids™ and dressings.

The term "dressing" refers broadly to any material applied to a wound for protection, absorbance, drainage, etc. Thus, adsorbent and absorbent materials are specifically contemplated as a solid support. Numerous types of dressings are commercially available, including films (*e.g.*, polyurethane films), hydrocolloids (hydrophilic colloidal particles bound to polyurethane foam), hydrogels (cross-linked polymers containing about at least 60% water), foams (hydrophilic or hydrophobic), calcium alginates (nonwoven composites of fibers from calcium alginate), and cellophane (cellulose with a plasticizer) [Kannon and Garrett, *Dermatol. Surg.* 21:583-590 (1995); Davies, *Burns* 10:94 (1983)]. The present invention specifically contemplates the use of dressings impregnated with the wound healing promoting and enhancing compounds of the present invention.

The term "biocompatible" means that there is minimal (*i.e.*, no significant difference is seen compared to a control), if any, effect on the surroundings. For example, in some embodiments of the present invention, the dressing comprises a biocompatible membrane.

The term "fibronectin-derived peptide" means a peptide that is smaller than the intact fibronectin protein but that has sequence identical (or at least 90% identical) to a portion of the natural fibronectin sequence. For example, the peptide PHSRN has a sequence that exists in a portion of the natural fibronectin; the peptide PHSCN, while not existing as a portion of the natural sequence is, by this definition, a fibronectin-derived peptide. Typically, the peptide will be between four and one hundred amino acids (although larger fragments of fibronectin are possible, including but not limited to fragments wherein additional non-fibronectin amino acid sequences have been added to the peptide). A preferred fibronectin-derived peptide is one lacking the RGD motif of fibronectin. In yet another embodiment, said peptide lacks the motif which binds the $\alpha 5\beta 1$ receptor.

The term "peptide derivative" refers to compound having an imino group (-NH-), and more particularly, a peptide bond. Peptides may be regared as substituted amides. Like the amide group, the peptide bond shows a high degree of resonance stabilization. The C-N single bond in the peptide linkage has typically about 40 percent double-bond character and the C=O double bond about 40 percent single-bond character. Peptide derivatives include (but are not limited to) "peptide analogs" which are herein defined as compounds that can be incorporated into polypeptide chains in place of the corresponding natural amino acids by the natural enzymes (i.e. incorporated by aminoacyl-tRNA synthetases. Examples of such analogues include (but are not limited to) p-fluorophenylalanine (an analog of phenylalanine) and ethionine and norleucine (analogs of methionine).

"Protecting groups" are those groups which prevent undesirable reactions (such as proteolysis) involving unprotected functional groups. In one embodiment, the present invention contemplates that the protecting group is an acyl or an amide. In one embodiment, the acyl is acetate. In another embodiment, the protecting group is a benzyl group. In another embodiment, the protecting group is a benzoyl group. The present invention also contemplates combinations of such protecting groups.

The term "Band-Aid™" is meant to indicate a relatively small adhesive strip comprising an adsorbent pad (such as a gauze pad) for covering minor wounds.

DESCRIPTION OF THE INVENTION

The present invention provides compounds useful in (I) the diagnosis and treatment of cancer as well as in (II) wound healing. In addition, the present invention provides compounds useful as (III) anti-coagulants, (IV) adjuvants, and (V) enhancers in gene therapy.

I. Diagnosis And Treatment Of Cancer

As a prelude to metastasis, it is believed that cancer cells proteolytically alter basement membranes underlying epithelia or the endothelial linings of blood and lymphatic vessels, invade through the defects created by proteolysis, and enter the circulatory or lymphatic systems to colonize distant sites. During this process, the secretion of proteolytic enzymes is coupled with increased cellular motility and altered adhesion. After their colonization of distant sites, metastasizing tumor cells proliferate to establish metastatic nodules.

As noted above, chemotherapeutic agents are currently employed to reduce the unrestricted growth of cancer cells, either prior to surgical removal of the tumor (neoadjuvant

therapy) or after surgery (adjuvant therapy). However, none of these methods has proved curative once metastasis has occurred. Since unrestricted invasive behavior is also a hallmark of metastatic tumor cells, methods for directly inhibiting tumor cell invasion and metastasis are needed.

A. Assays For Testing Tumor Invasion

Discovering how to inhibit the invasive behavior of tumor cells to intervene in the metastatic cascade first requires the development of assays with which to test tumor cell invasion *in vitro*. Two assay systems are contemplated for use in the method of the present invention to test the tumor cell invasion.

1. Fibronectin-Depleted Substrates

In one assay system, the present invention contemplates using fibronectin-depleted substrates. These are substrates that originally contain fibronectin that are treated according to the methods of the present invention (see below) to remove fibronectin. It is not intended that the present invention be limited by the nature of the original substrate; such fibronectin-containing substrates suitable for treatment and depletion include: i) complex substrates containing a variety of extracellular proteins and ii) less complex substrates containing fibronectin along with one or two other proteins (*e.g.* ,collagen, laminin, etc.).

It is also not intended that the present invention be limited by the precise amount of fibronectin remaining after the substrate has been treated. In other words, while the methods of the present invention remove fibronectin, and in some embodiments. remove substantially all fibronectin, it is within the meaning of the term "fibronectin-depleted" substrate that a small amount of fibronectin remain in the substrate.

In one embodiment, the present invention contemplates using an extracellular matrix available commercially. For example, the present invention contemplates treating basement membrane matrices such as ECM GEL, a matrix from mouse sarcoma (commercially available from Sigma, St. Louis, Mo). However, it is not intended that the present invention be limited by the particular fibronectin-containing substrate. For example, other commercially available substrates are contemplated, such as the commonly used substrate Matrigel (available from Becton Dickinson Labware, Catalog #40234); Matrigel can be treated appropriately according to the methods of the present invention so as to render it "fibronectin-depleted" (see below). Untreated Matrigel (and similar substrates) have been used to demonstrate the

importance of proteases and motility factors in the invasion and metastasis of many tumors. However, these invasion substrates are not available as serum-free substrates; thus, the regulation of tumor cell invasive behavior by serum components, such as plasma fibronectin, is a complicating factor with untreated Matrigel.

Consequently, the present invention contemplates a fibronectin-free substrate. In this embodiment, Matrigel is treated so that it is substantially fibronectin-free. The preparation of fibronectin-free Matrigel involves "panning" the Matrigel substrate on gelatin as well as "panning" the substrate on anti-fibronectin antibody (anti-human fibronectin IgG is available commercially, such as antibody from Promega Corporation, Madison, Wisconsin).

2. Naturally Occurring Fibronectin-Free Substrates

In another embodiment, the present invention contemplates substrates that are naturally free of fibronectin; such a source provides, for example, basement membranes permeable to select types of normally invasive cells, such membranes being naturally serum-free. In one embodiment, the present invention contemplates sea urchins as a source of such membranes. In this regard, the ectoderm of sea urchin embryos is one cell thick, and secretes an underlying basement membrane (see Figure 1) very similar to that of mammals. These embryos contain no circulatory or lymphatic systems; and thus, their basement membranes are serum-free. In embryos, the subectodermal basement membrane functions simultaneously as a migration substrate for several, specific mesenchymal cell types while it functions as an invasion substrate for others. Sea urchin embryo basement membranes (SU-ECM) can be prepared by mild detergent treatment as described in D. Livant *et al.*, *Cancer Research* 55:5085 (1995) and described in the Experimental section below.

Regardless of which of the two types of substrates are employed, the invasion substrates of the present invention are easy to prepare and give rapid, highly consistent results with a variety of cells, including: a) cell lines from: i) primary and metastatic tumors, and ii) normal epithelial tissues; as well as b) cells from primary tissue samples of both tumors, their surrounding normal tissues, and neonatal melanocytes, fibroblasts, and keratinocytes from circumcised tissue.

In one embodiment, the present invention contemplates a method of evaluating human cancer comprising: a) providing: i) a human cancer patient (such as a patient with breast cancer or prostate cancer), ii) a fibronectin-free substrate (for example, a fibronectin-depleted substrate) and iii) one or more invasion-inducing agents (discussed below); b) obtaining

cancer cells from said patient (such as from a biopsy); c) contacting said cells *ex vivo* (*i.e.*, outside the body) with said fibronectin-free substrate and said one or more invasion-inducing agents; and d) measuring the extent of cancer cell invasion of said substrate. Preferably the cancer cells are cultured (or at least briefly maintained) in serum-free culture media prior to testing for invasion so as to avoid introducing complicating serum factors.

3. Inducing Agents

It is not intended that the present invention be limited by the nature of the agent that causes or induces cells to invade the fibronectin-free substrates of the present invention. Such agents can be identified functionally by simply adding them to the cell culture and measuring the extent of invasion.

In one embodiment, the invasion-inducing agent comprises a peptide derived from fibronectin. In a preferred embodiment, the invasion inducing agent is intact fibronectin.

While not limited to any mechanism, it is believed that cells exposed to invasion-inducing agents in this manner are potentially rendered capable of invading the substrate. Again, while not limited to any mechanism, it is believed that the invasion inducing agent comprising the sequence PHSRN binds to the $\alpha 5\beta 1$ receptor on the cancer cell and thereby induces invasion of the substrate. In this regard, the present invention provides a method of treating cells comprising: a) providing: i) cells expressing the $\alpha 5\beta 1$ receptor, ii) a fibronectin-free substrate, and iii) one or more invasion-inducing agents; b) culturing (or least maintaining) said cells in serum-free culture media on said substrate in the presence of said invasion-inducing agents; and d) measuring the extent of cell invasion of said substrate. In one embodiment, the cells are normal epithelial cells or fibroblasts. In another embodiment, the cells are human cancer cells.

B. Drug Screening Assays

The present invention also contemplates a screening assay for identifying drugs that inhibit tumor invasion. The present invention contemplates a screening assay (in the presence and absence of serum) utilizing the binding activity of fibronectin-derived peptides. In one embodiment, an inducible tumor cell line is placed in tissue culture on a fibronectin-free substrate. The tumor cells will be induced (under ordinary conditions) by the fibronectin-derived peptide to invade the substrate.

In one embodiment, the invasion-inducing agent comprises a peptide derived from fibronectin. In a preferred embodiment, said peptide comprises the sequence PHSRN. Of course, the peptide may be larger than five amino acids; indeed, the peptide fragment of fibronectin may contain hundreds of additional residues (*e.g.*, five hundred amino acids). One such larger peptide is set forth in U.S. Patent 5,492,890 (hereby incorporated by reference). In one embodiment, the PHSRN-containing peptide is less than one hundred amino acids in length and lacks the RGD sequence characteristic of fibronectin. A variety of PHSRN-containing peptides are contemplated, including the PHSRN peptide itself and related peptides where additional amino acids are added to the carboxyl terminus, including (but not limited to) peptides comprising the sequence: 1) PHSRN, 2) PHSRNS, 3) PHSRNSI, 4) PHSRNSIT, 5) PHSRNSITL, 6) PHSRNSITLT, 7) PHSRNSITLTN, 8) PHSRNSITLTNL, 9) PHSRNSITLTNLT, 10) PHSRNSITLTNLTP, and 11) PHSRNSITLTNLTPG. Alternatively, PHSRN-containing peptides are contemplated where amino acids are added to the amino terminus, including (but not limited to) peptides comprising the sequence: 1) PEHFSGRPREDRVPHSRN, 2) EHFSGRPREDRVPHSRN, 3) HFSGRPREDRVPHSRN, 4) FSGRPREDRVPHSRN, 5) SGRPREDRVPHSRN, 6) GRPREDRVPHSRN, 7) RPREDRVPHSRN, 8) PREDRVPHSRN, 9) REDRVPHSRN, 10) EDRVPHSRN, 11) DRVPHSRN, 12) RVPHSRN, and 13) VPHSRN. Finally, the present invention contemplates PHSRN-containing peptides where amino acids are added to both the amino and carboxyl termini, including (but not limited to) peptides comprising the sequence PEHFSGRPREDRVPHSRNSITLTNLTPG, as well as peptides comprising portions or fragments of the PHSRN-containing sequence PEHFSGRPREDRVPHSRNSITLTNLTPG.

Peptides containing variations on the PHSRN motif are contemplated. For example, the present invention also contemplates PPSRN-containing peptides for use in the above-named assays. Such peptides may vary in length in the manner described above for PHSRN-containing peptides. Alternatively, PPSRN may be used as a peptide of five amino acids.

Similarly, peptides comprising the sequence -HHSRN-, -HPSRN-, -PHTRN-, -HHTRN-, -HPTRN-, -PHSNN-, -HHSNN-, -HPSNN-, -PHTNN-, -HHTNN-, -HPTNN-, -PHSKN-, -HHSKN-, -HPSKN-, -PHTKN-, -HHTKN-, -HPTKN-,-PHSRR-, -HHSRR-, -HPSRR-, -PHTRR-, -HHTRR-, -HPTRR-, -PHSNR-, -HHSNR-, -HPSNR-, -PHTNR-, -HHTNR-, -HPTNR-, -PHSKR-, -HHSKR-, -HPSKR-, -PHTKR-, -HHTKR-, -HPTKR-, -PHSRK-, -HHSRK-, -HPSRK-, -PHTRK-, -HHTRK-, -HPTRK-, -PHSNK-, -HHSNK-, -HPSNK-, -PHTNK-, -HHTNK-, -HPTNK-, -PHSKK-, -HHSKK-, -HPSKK-, -PHTKK-, -

HHTKK-, or -HPTKK- are contemplated by the present invention. Such peptides can be used as five amino acid peptides or can be part of a longer peptide (in the manner set forth above for PHSRN-containing peptides).

In another embodiment, the present invention contemplates an inducing agent comprising the amino acid sequence $X_1X_2X_3X_4X_5$, wherein X_1 is an amino acid selected from the group consisting of proline, glycine, valine, histidine, isoleucine, phenylalanine, tyrosine, and tryptophan, and X_2 is an amino acid selected from the group consisting of histidine, proline, tyrosine, asparagine, glutamine, arginine, lysine, glycine, phenylalanine, and tryptophan, and X_3 is an amino acid selected from the group consisting of serine, threonine, alanine, tyrosine, leucine, histidine, asparagine, glycine and glutamine, and X_4 is an amino acid selected from the group consisting of arginine, lysine, and histidine, and X_5 is an amino acid selected from the group consisting of asparagine, glutamine, serine, threonine, histidine, glycine and tyrosine.

In this drug screening assay, candidate drug inhibitors are added to the tissue culture (this can be done individually or in mixtures). Where the inducible tumor cell is found to be inhibited from invading the substrate, a drug inhibitor is indicated (see Examples section below using the PHSCN peptide).

It is not intended that the present invention be limited by the nature of the drugs screened in the screening assay of the present invention. A variety of drugs, including peptides and non-peptide mimetics, are contemplated.

It is also not intended that the present invention be limited by the particular tumor cells used for drug testing. A variety of tumor cells (for both positive and negative controls) are contemplated (including but not limited to the cells set forth in Table 1 below).

C. Invasion-Inducing Agents And Antagonists

While an understanding of the mechanisms involved in metastatic cancer is not necessary to the successful practice of the present invention, it is believed that tumor cell invasion of basement membranes occurs at several points in the metastatic cascade: (1) when epithelial tumor cells (such as those of breast and prostate cancers) leave the epithelium and enter the stroma, (2) when tumor cells enter the circulatory or lymphatic systems, and (3) when tumor cells leave the circulatory or lymphatic systems to invade distant sites. Thus, intervention in the *induction* of tumor cell invasiveness by using a PHSRN antagonist, such as

the PHSCN peptide, to block tumor cell receptors for this sequence is contemplated as a method for decreasing the rate of metastasis.

One advantage of this strategy is that leukocytes are the only normal cells known to invade tissues routinely to carry out their functions, and relatively few leukocytes are invasive at a given time. Thus, relatively small doses of an anti-invasion antagonist which blocks the binding of PHSRN to its receptor are required. Also, other than some immunodepression, there should be relatively few side effects associated with anti-metastatic treatment using compounds designed to block the induction of invasion. The lack of debilitating side effects expected from anti-invasive therapy means that using it in combination with anti-proliferative agents would be uncomplicated, and that it could be used prior to surgery or even prophylactically to block tumor cell invasion and metastasis.

The IKVAV sequence of laminin, a prevalent insoluble protein of the extracellular matrix, is known to stimulate liver colonization by metastatic human colon cancer cells in athymic mice [*see* Bresalier *et al.*, *Cancer Research* 55:2476 (1995)]. Since IKVAV, like PHSRN, contains a basic amino acid (K) which, by virtue of its positive charge, might also function to displace a divalent cation from its integrin receptor and stimulate invasion,the present invention contemplates applying the strategy of developing anti-invasion antagonists to the IKVAV sequence of laminin.

TABLE 1

Designation And Origin Of Human Cell Lines And Strains[1]

Origin	Cell Lines or Strains
Colonic carcinoma	SW1116, HCT116, SKCO-1, HT-29, KM12C, KM12SM, KM12L4, SW480
Pancreatic carcinoma	BxPC-3, AsPC-1, Capan-2, MIA PaCa-2, Hs766T
Colon adenoma	VaCo 235
Lung carcinoma	A549
Prostate carcinoma	PC-3, DU-145
Breast carcinoma	009P, 013T, SUM-52 PE
Lymphoma	Daudi, Raji
Breast epithelium	006FA
Diploid fibroblast	HCS (human corneal stroma), MRC-5

[1] The SW1116, HT-29, SW480, Raji lymphoblastoid cells, and the pancreatic lines are obtained from the American Type Culture Collection.

1. Antagonists

It is not intended that the present invention be limited by the nature of the agent that inhibits tumor invasiveness. A variety of anti-invasive chemotherapeutics are contemplated to antagonize the invasion-promoting activity of the PHSRN sequence.

In the preferred embodiment, the anti-invasive agent is a peptide with the amino acid sequence PHSCN. In another embodiment, the anti-invasive agent is a peptide which has an amino acid sequence comprising a sequence selected from the group consisting of CHSRN, PCSRN, PHCRN, and PHSRC. In another embodiment, the anti-invasive agent is a peptide which has an amino acid sequence comprising PHSXN, where X is an amino acid selected from the group consisting of homo-cysteine, the D-isomer of cysteine, histidine, and penicillamine.

The present invention also contemplates an anti-invasive agent comprising the amino acid sequence X_1HSX_2N, wherein X_1 is either proline, histidine, an amino acid analogue or not an amino acid, and X_2 is an amino acid selected from the group consisting of the L-isomer of cysteine, the D-isomer of cysteine, homo-cysteine, histidine, and penicillamine. In another embodiment, the present invention contemplates an anti-invasive agent comprising the amino acid sequence $X_1X_2X_3X_4X_5$, wherein X_1 is an amino acid selected from the group consisting of proline, glycine, valine, histidine, isoleucine, phenylalanine, tyrosine, and tryptophan, and X_2 is an amino acid selected from the group consisting of histidine, proline, tyrosine, glycine asparagine, glutamine, arginine, lysine, phenylalanine, and tryptophan, and X_3 is an amino acid selected from the group consisting of serine, threonine, alanine, tyrosine, glycine, leucine, histidine, asparagine, and glutamine, and X_4 is an amino acid selected from the group consisting of cysteine, homo-cysteine, penicillamine, histidine, tyrosine, asparagine, glutamine, and methionine, and X_5 is an amino acid selected from the group consisting of asparagine, glutamine, serine, threonine, histidine, glycine and tyrosine. In the preferred embodiment the peptide is PHSCN, where the cysteine is either the L-isomer or D-isomer.

Similarly, peptides comprising the sequence -PSCN-, -HSCN-, -PSCN-, -HTCN-, -PTCN-, -HSCN-, -HSCN-, -PSCN-, -HTCN-, HTCN-, -PTCN-, -HSCN-, -HSCN-, -PSCN-, -HTCN-, -HTCN-, -PTCN-, -HSCR-, -HSCR-, -PSCR-, -HTCR-, -HTCR-, -PTCR-, -HSCR-, -HSCR-, -PSCR-, -HTCR-, -HTCR-, -PTCR-, -HSCR-, -HSCR-, -PSCR-, -HTCR-, -HTCR-, -PTCR-, -HSCK-, -HSCK-, -PSCK-, -HTCK-, -HTCK-, -PTCK-, -HSCK-, -HSCK-, -PSCK-, -HTCK-, -HTCK-, -PTCK-, -HSCK-, -HSCK-, -PSCK-, -HTCK-, -HTCK-, or -PTCK- are contemplated by the present invention.

It is further contemplated that, in some embodiments, the anti-invasive agents named above comprise the named amino acid sequence and additional amino acids added to the amino terminus, the carboxyl terminus, or both the amino and carboxyl termini (in the manner set forth above for the PHSRN containing peptides, *e.g.*, PHSRNSIT). In one embodiment, the anti-invasive agent is up to five hundred amino acids in length. It is also contemplated that, in some embodiments, the anti-invasive agents named above comprise a peptide with the amino terminus blocked by standard methods to prevent digestion by exopeptidases, for example by acetylation; and the carboxyl terminus blocked by standard methods to prevent digestion by exopeptidases, for example, by amidation.

In this regard, the present invention provides a method of treating cancer comprising: a) providing: i) a subject having cancer, and ii) a composition of matter comprising a peptide, peptide derivative, or peptide mimetic which inhibits the tumor invasion-promoting activity of a peptide comprising the amino acid sequence PHSRN, and b) administering said composition to said subject. The present invention further contemplates using antagonists before and/or after surgical removal of the primary tumor. In one embodiment, the method comprises administering a PHSRN antagonist as adjunct therapy with additional chemotherapeutics.

While not limited to any mechanism, it is believed that these anti-invasive chemotherapeutic agents antagonize the invasion-promoting activity of the PHSRN sequence (*e.g.*, of fibronectin) by blocking the binding of this sequence to its receptor on tumor cells. Again, while not limited to any mechanism, it is believed that the PHSRN sequence may promote invasion by acting to displace a divalent cation (Mg+2, Ca+2, or Mn+) in the $\alpha 5\beta 1$ receptor on metastatic tumor cells, and the above named chemotherapeutic anti-invasive agents might act to inhibit this invasion by chelating one or more of these divalent cations.

In another embodiment, the present invention contemplates anti-invasion antagonists to the IKVAV sequence of laminin.

2. Designing Mimetics

Compounds mimicking the necessary conformation for recognition and docking to the receptor binding to the peptides of the present invention are contemplated as within the scope of this invention. For example, mimetics of PHSRN and PHSRN-antagonists are contemplated. A variety of designs for such mimetics are possible. For example, cyclic PHSRN and PHSCN containing peptides, in which the necessary conformation for binding is

stabilized by nonpeptides, are specifically contemplated. United States Patent No. 5,192,746 to Lobl, *et al.*, United States Patent No. 5,169,862 to Burke, Jr., *et al.*, United States Patent No. 5,539,085 to Bischoff, *et al.*, United States Patent No. 5,576,423 to Aversa, *et al.*, United States Patent No. 5,051,448 to Shashoua, and United States Patent No. 5,559,103 to Gaeta, *et al.*, all hereby incorporated by reference, describe multiple methods for creating such compounds.

Synthesis of nonpeptide compounds that mimic peptide sequences is also known in the art. Eldred, *et al.*, (*J. Med. Chem.* 37:3882 (1994)) describe nonpeptide antagonists that mimic the Arg-Gly-Asp sequence. Likewise, Ku, *et al.*, (*J. Med. Chem.* 38:9 (1995)) give further elucidation of the synthesis of a series of such compounds. Such nonpeptide compounds that mimic PHSRN and PHSRN-antagonists are specifically contemplated by the present invention.

The present invention also contemplates synthetic mimicking compounds that are multimeric compounds that repeat the relevant peptide sequences. In one embodiment of the present invention, it is contemplated that the relevant peptide sequence is Pro-His-Ser-Arg-Asn; in another embodiment, the relevant peptide sequence is Pro-His-Ser-Cys-Asn; in another embodiment, the relevant peptide sequence is Ile-Lys-Val-Ala-Val. As is known in the art, peptides can be synthesized by linking an amino group to a carboxyl group that has been activated by reaction with a coupling agent, such as dicyclohexylcarbodiimide (DCC). The attack of a free amino group on the activated carboxyl leads to the formation of a peptide bond and the release of dicyclohexylurea. It can be necessary to protect potentially reactive groups other than the amino and carboxyl groups intended to react. For example, the α-amino group of the component containing the activated carboxyl group can be blocked with a tertbutyloxycarbonyl group. This protecting group can be subsequently removed by exposing the peptide to dilute acid, which leaves peptide bonds intact. With this method, peptides can be readily synthesized by a solid phase method by adding amino acids stepwise to a growing peptide chain that is linked to an insoluble matrix, such as polystyrene beads. The carboxyl-terminal amino acid (with an amino protecting group) of the desired peptide sequence is first anchored to the polystyrene beads. The protecting group of the amino acid is then removed. The next amino acid (with the protecting group) is added with the coupling agent. This is followed by a washing cycle. The cycle is repeated as necessary.

In one embodiment, the mimetics of the present invention are peptides having sequence homology to the above-described PHSRN sequences and PHSRN-antagonists. One

common methodology for evaluating sequence homology, and more importantly statistically significant similarities, is to use a Monte Carlo analysis using an algorithm written by Lipman and Pearson to obtain a Z value. According to this analysis, a Z value greater than 6 indicates probable significance, and a Z value greater than 10 is considered to be statistically significant. W.R. Pearson and D.J. Lipman, Proc. Natl. Acad. Sci. (USA), 85:2444-2448 (1988); D.J. Lipman and W.R. Pearson, Science, 227:1435-1441 (1985). In the present invention, synthetic polypeptides useful in tumor therapy and in blocking invasion are those peptides with statistically significant sequence homology and similarity (Z value of Lipman and Pearson algorithm in Monte Carlo analysis exceeding 6).

3. Antibody Inhibitors

The present invention contemplates all types of inhibitors of tumor invasion for use in both the assays and for therapeutic use. In one embodiment, the present invention contemplates antibody inhibitors. The antibodies may be monoclonal or polyclonal, but polyclonal antibodies are often more effective inhibitors. It is within the scope of this invention to include any second antibodies (monoclonal or polyclonal) directed to the first antibodies discussed above. Both the first and second antibodies may be used in the detection assays or a first antibody may be used with a commercially available anti-immunoglobulin antibody. An antibody as contemplated herein includes any antibody specific to any region of a peptide involved in the induction of tumor cell invasion. For example, the present invention contemplates antibodies reactive with PHSRN peptides (as well as the related peptides set forth above).

Both polyclonal and monoclonal antibodies are obtainable by immunization with peptides, as well as with enzymes or proteins, and all types are utilizable for immunoassays. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of the purified enzyme or protein, or antigenic parts thereof, collecting serum from the animal, and isolating specific sera by any of the known immunoadsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of immunoassay, they are generally less favored because of the potential heterogeneity of the product.

The use of monoclonal antibodies in an immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The

preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art. (See, for example Douillard and Hoffman, Basic Facts about Hybridomas, in Compendium of Immunology Vol II, ed. by Schwartz, 1981; Kohler and Milstein, Nature 256: 495-499, 1975; European Journal of Immunology 6: 511-519, 1976).

Unlike preparation of polyclonal sera, the choice of animal is dependent on the availability of appropriate immortal lines capable of fusing with lymphocytes. Mouse and rat have been the animals of choice in hybridoma technology and are preferably used. Humans can also be utilized as sources for sensitized lymphocytes if appropriate immortalized human (or nonhuman) cell lines are available. For the purpose of the present invention, the animal of choice may be injected with an antigenic amount, for example, from about 0.1 mg to about 20 mg of the enzyme or protein or antigenic parts thereof. Usually the injecting material is emulsified in Freund's complete adjuvant. Boosting injections may also be required. The detection of antibody production can be carried out by testing the antisera with appropriately labelled antigen. Lymphocytes can be obtained by removing the spleen of lymph nodes of sensitized animals in a sterile fashion and carrying out fusion. Alternatively, lymphocytes can be stimulated or immunized in vitro, as described, for example, in Reading, Journal of Immunological Methods 53: 261-291, 1982.

A number of cell lines suitable for fusion have been developed and the choice of any particular line for hybridization protocols is directed by any one of a number of criteria such as speed, uniformity of growth characteristics, deficiency of its metabolism for a component of the growth medium, and potential for good fusion frequency.

Intraspecies hybrids, particularly between like strains, work better than interspecies fusions. Several cell lines are available, including mutants selected for the loss of ability to secrete myeloma immunoglobulin.

Cell fusion can be induced either by virus, such as Epstein-Barr or Sendai virus, or polyethylene glycol. Polyethylene glycol (PEG) is the most efficacious agent for the fusion of mammalian somatic cells. PEG itself may be toxic for cells and various concentrations should be tested for effects on viability before attempting fusion. The molecular weight range of PEG may be varied from 1000 to 6000. It gives best results when diluted to from about 20% to about 70% (w/w) in saline or serum-free medium. Exposure to PEG at 37°C for about 30 seconds is preferred in the present case, utilizing murine cells. Extremes of

temperature (*i.e.*, about 45°C) are avoided, and preincubation of each component of the fusion system at 37°C prior to fusion can be useful. The ratio between lymphocytes and malignant cells is optimized to avoid cell fusion among spleen cells and a range of from about 1:1 to about 1:10 is commonly used.

The successfully fused cells can be separated from the myeloma line by any technique known by the art. The most common and preferred method is to choose a malignant line which is Hypoxthanine Guanine Phosphoribosyl Transferase (HGPRT) deficient, which will not grow in an aminopterin-containing medium used to allow only growth of hybrids and which is generally composed of hypoxthanine 1x10-4M, aminopterin 1x10-5M, and thymidine 3x10-5M, commonly known as the HAT medium. The fusion mixture can be grown in the HAT-containing culture medium immediately after the fusion 24 hours later. The feeding schedules usually entail maintenance in HAT medium for two weeks and then feeding with either regular culture medium or hypoxthanine, thymidine-containing medium.

The growing colonies are then tested for the presence of antibodies that recognize the antigenic preparation. Detection of hybridoma antibodies can be performed using an assay where the antigen is bound to a solid support and allowed to react to hybridoma supernatants containing putative antibodies. The presence of antibodies may be detected by "sandwich" techniques using a variety of indicators. Most of the common methods are sufficiently sensitive for use in the range of antibody concentrations secreted during hybrid growth.

Cloning of hybrids can be carried out after 21-23 days of cell growth in selected medium. Cloning can be preformed by cell limiting dilution in fluid phase or by directly selecting single cells growing in semi-solid agarose. For limiting dilution, cell suspensions are diluted serially to yield a statistical probability of having only one cell per well. For the agarose technique, hybrids are seeded in a semi-solid upper layer, over a lower layer containing feeder cells. The colonies from the upper layer may be picked up and eventually transferred to wells.

Antibody-secreting hybrids can be grown in various tissue culture flasks, yielding supernatants with variable concentrations of antibodies. In order to obtain higher concentrations, hybrids may be transferred into animals to obtain inflammatory ascites. Antibody-containing ascites can be harvested 8-12 days after intraperitoneal injection. The ascites contain a higher concentration of antibodies but include both monoclonals and immunoglobulins from the inflammatory ascites. Antibody purification may then be achieved by, for example, affinity chromatography.

A wide range of immunoassay techniques are available for evaluating the antibodies of the present invention as can be seen by reference to US Patent Nos. 4,016,043; 4,424,279 and 4,018,653, hereby incorporated by reference. This, of course, includes both single-site and two-site, or "sandwich", assays of the non-competitive types, as well as in the traditional competitive binding assays.

4. Administering Chemotherapeutics

It is contemplated that the antagonists of the present invention be administered systemically or locally to inhibit tumor cell invasion in cancer patients with locally advanced or metastatic cancers. They can be administered intravenously, intrathecally, intraperitoneally as well as orally. PHSRN antagonists (*e.g.*, the PHSCN peptide), can be administered alone or in combination with anti-proliferative drugs in a neoadjuvant setting to reduce the metastatic load in the patient prior to surgery; or they can be administered after surgery. Since PHSRN antagonists may depress wound healing (because the PHSRN sequence also elicits fibroblast invasion as described below), it may be necessary to use PHSRN antagonists some time after surgery to remove the tumor.

Since few cells in the body must invade in order to function, PHSRN antagonists administered systemically are not likely to cause the debilitating side effects of cytotoxic chemotherapeutic agents. However, since they suppress invasion, they are likely to cause some immunodepression. Even so, at the appropriate dosage, PSHRN antagonists may be administered prophylactically. In any case, it is contemplated that they may be administered in combination with cytotoxic agents. The simultaneous selection against the two fatal attributes of metastatic cells, unrestricted proliferation and invasion, is contemplated as a very powerful therapeutic strategy.

Where combinations are contemplated, it is not intended that the present invention be limited by the particular nature of the combination. The present invention contemplates combinations as simple mixtures as well as chemical hybrids. An example of the latter is where the antagonist is covalently linked to a targeting carrier or to an active pharmaceutical. Covalent binding can be accomplished by any one of many commercially available crosslinking compounds.

It is not intended that the present invention be limited by the particular nature of the therapeutic preparation. For example, such compositions can be provided together with physiologically tolerable liquid, gel or solid carriers, diluents, adjuvants and excipients.

These therapeutic preparations can be administered to mammals for veterinary use, such as with domestic animals, and clinical use in humans in a manner similar to other therapeutic agents. In general, the dosage required for therapeutic efficacy will vary according to the type of use and mode of administration, as well as the particularized requirements of individual hosts.

Such compositions are typically prepared as liquid solutions or suspensions, or in solid forms. Oral formulations for cancer usually will include such normally employed additives such as binders, fillers, carriers, preservatives, stabilizing agents, emulsifiers, buffers and excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders, and typically contain 1%-95% of active ingredient, preferably 2%-70%.

The compositions are also prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared.

The antagonists of the present invention are often mixed with diluents or excipients which are physiological tolerable and compatible. Suitable diluents and excipients are, for example, water, saline, dextrose, glycerol, or the like, and combinations thereof. In addition, if desired the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, stabilizing or pH buffering agents.

Additional formulations which are suitable for other modes of administration, such as topical administration, include salves, tinctures, creams, lotions, and, in some cases, suppositories. For salves and creams, traditional binders, carriers and excipients may include, for example, polyalkylene glycols or triglycerides.

II. Promoting And Enhancing Wound Healing

As noted above, it is contemplated that PHSRN antagonists may depress wound healing. This expectation is based on the discovery that PHSRN-containing peptides promote wound healing.

In this regard, it should be noted that the therapy of wounds, particularly those which are made difficult to heal by disease, has been attempted with a variety of purified growth factors or cytokines because these molecules can induce cellular proliferation or increase the

motility of cells in wounds. Thus, if presented in the correct form and location at the right time, growth factors may greatly accelerate or enhance the healing of wounds by stimulating the growth of new tissue. Given the complexity and clinical variability of wounds, an obvious difficulty with the application of specific, purified growth factors or cytokines to wounded tissue, alone or in combination, is that their forms or specific distributions in the wound may not support their normal activities. Instead, the effectiveness of growth factors and cytokines in promoting the healing of wounded tissue may depend on their secretion by fibroblasts or macrophages.

The present invention contemplates a more effective approach; this approach involves methods that stimulate the invasion of the wound by the cells which synthesize the growth factors and cytokines active in stimulating wound repair, especially monocytes, macrophages, and fibroblasts. This strategy allows the cells in their normal *in vivo* setting to secrete the active factors. This approach has a number of advantages: (1) the temporal and spatial distributions of the factors are likely to be optimal because the normally active cells in their correct settings are secreting them; (2) all the appropriate factors are likely to be present in their active forms, irrespective of whether they have been identified or cloned; (3) the sequential effects of the factors in recruiting subsequent waves of cells involved in the healing process to the wound site are likely to be enhanced by the presence of more initiating cells in the wound.

The present invention is based on the discovery that the pure PHSRN peptide or purified plasma fibronectin fragments containing it, and lacking the $\alpha 4\beta 1$ integrin binding site in the IIICS region, are sufficient to stimulate fibroblast invasion of basement membranes *in vitro* in the presence of serum or under serum-free conditions, while intact plasma fibronectin fails to stimulate fibroblast invasion. Pure PHSRN peptide has also been shown to stimulate keratinocyte invasion of serum-free SU-ECM. Since, during wound reepithelialization, keratinocytes migrate through the connective tissue of the provisional matrix to "wall off" portions of the wound, as well as through the adjacent stroma, it is not surprising that they are also stimulated to migrate through the matrix of SU-ECM invasion substrates by the PHSRN sequence.. This suggests that this peptide, or proteinase-resistant forms of it, may have similar effects on fibroblasts, keratinocytes, and monocytes/macrophages *in vivo*. Recruitment of fibroblasts or monocytes/macrophages whose paracrine, regulatory effects on a variety of neighboring cells are required for the early stages of wound healing is contemplated as a highly efficient and effective way to stimulate the cascade of regulatory interactions involved

in wound healing because these cells will secrete the active factors or cytokines in the correct temporal sequences and spatial locations to ensure their optimal activities. Because it efficiently induces keratinocyte migration through the extracellular matrix *in vitro*, the PHSRN peptide is also likely to stimulate wound reepithelialization directly. The use of the PHSRN peptide or structurally related molecules according to the present invention is to stimulate the entry of cells such as fibroblasts and monocyte/macrophages into the provisional matrix of a wound, so that the entering cells themselves secrete the factors and cytokines active in inducing or potentiating wound healing. The use of the PHSRN peptide or structurally related molecules is also intended to stimulate wound reepithelialization directly by inducing keratinocyte migration through the extracellular matrix.

The present invention contemplates 1) assays to rapidly and readily assess both a) the invasion potential of cells of patients (such as burn or diabetes patients) as well as b) the potential of candidate inducing agents (such as non-peptide compounds having similar activity to PHSRN-containing peptides) and 2) compositions and methods for the treatment of patients with wounds.

A. Assays For Testing Invasion Potential And Screening New Therapeutics

It may be desirable to test the potential of cells for invasion, thereby predicting the ability of a patient to respond to the wound treatment according to the present invention. Similarly, it may be desirable to screen new potential therapeutics for their level of inducing activity. Two assay systems are contemplated for such testing.

1. Fibronectin-depleted Substrates

In one assay system, the present invention contemplates using fibronectin-depleted substrates. These are substrates that originally contain fibronectin that are treated according to the methods of the present invention (see below) to remove fibronectin. It is not intended that the present invention be limited by the nature of the original substrate; such fibronectin-containing substrates suitable for treatment and depletion include i) complex substrates containing a variety of extracellular proteins and ii) less complex substrates containing fibronectin along with one or two other proteins (*e.g.* collagen, laminin, etc.).

It is also not intended that the present invention be limited by the precise amount of fibronectin remaining after the substrate has been treated. In other words, while the methods

of the present invention remove fibronectin, and in some embodiments, remove substantially all fibronectin, it is within the meaning of the term "fibronectin-depleted" substrate that a small amount of fibronectin remain in the substrate.

In one embodiment, the present invention contemplates using an extracellular matrix available commercially. For example, the present invention contemplates treating basement membrane matrices such as ECM GEL, a matrix from mouse sarcoma (commercially available from Sigma, St. Louis, Mo). However, it is not intended that the present invention be limited by the particular fibronectin-containing substrate. For example, other commercially available substrates are contemplated, such as the commonly used substrate Matrigel (available from Becton Dickinson Labware, Catalog #40234); Matrigel can be treated appropriately according to the methods of the present invention so as to render it "fibronectin-depleted" (see below).

Consequently, the present invention contemplates a fibronectin-free substrate. In this embodiment, Matrigel is treated so that it is substantially fibronectin-free. The preparation of fibronectin-free Matrigel involves "panning" the Matrigel substrate on gelatin as well as "panning" the substrate on anti-fibronectin antibody (anti-human fibronectin IgG is available commercially, such as antibody from Promega Corporation, Madison, Wisconsin).

2. Naturally Occurring Fibronectin-free Substrates

In another embodiment, the present invention contemplates substrates that are naturally free of fibronectin; such a source provides, for example, basement membranes permeable to select types of normally invasive cells, such membranes being naturally serum-free. In one embodiment, the present invention contemplates sea urchins as a source of such membranes. In this regard, the ectoderm of sea urchin embryos is one cell thick, and secretes an underlying basement membrane (see Figure 1) very similar to that of mammals. These embryos contain no circulatory or lymphatic systems; and thus, their basement membranes are serum-free. In embryos, the subectodermal basement membrane functions simultaneously as a migration substrate for several, specific mesenchymal cell types while it functions as an invasion substrate for others.

Sea urchin embryo basement membranes (SU-ECM) can be prepared by mild detergent treatment as described in D. Livant *et al.*, *Cancer Research* 55:5085 (1995). Briefly, adult *Strongylocentrotus purpuratus* sea urchins can be obtained commercially (*e.g.* from Pacific BioMarine), and their embryos cultured to the early pluteus stage in artificial sea

water at 15°C. SU-ECM are then prepared from them by treatment with nonionic detergent and strerilized by dilution in the appropriate media.

Cells for the invasion assay are harvested by rinsing in Hanks' balanced salt solution, followed by brief treatment with 0.25% trypsin, 0.02% EDTA, and pelleting and resuspension in the appropriate medium with or without 5% FCS at a density of about 50,000 cells per ml. When appropriate, purified bovine plasma fibronectin (Sigma), purified 120 kDa chymotryptic fragment (Gibco BRL) or PHSRN peptides (synthesized at the Biomedical Research Core Facilities of the University of Michigan) are added to the resuspended cells prior to placement of the cells on SU-ECM. In each well of a plate used for an invasion assay, SU-ECM were placed in 0.5 ml of the appropriate medium, and 0.5ml of the resuspended cells dropped on their exterior surfaces. Invasion assays were incubated 1 to 16 hours prior to assay. If some circumstances, invasion assays were fixed in phosphate-buffered saline (PBS) with 2% formaldehyde for 5 minutes at room temperature, then rinsed into PBS.

Invasion assays are coded and scored blindly by microscopic examination under phase contrast at 200- and 400-fold magnification. Each cell contacting an SU-ECM is scored for its position relative to the exterior or interior surfaces. A cell is judged to have invaded if it is located on an interior surface below the focal plane passing through the upper surface of the SU-ECM, but above the focal plane passing through its lower surface. The minimum viability of the cells in each assay is always ascertained at the time of assay by determining the fraction of spread, adherent cells on the bottom of each well scored.

An invasion frequency is defined as the fraction of cells in contact with basement membranes which are located in their interiors at the time of assay. Thus, an invasion frequency of 1 denotes invasion by 100% of the cells in contact with basement membranes. Invasion frequencies are determined multiple times for each cell type assayed. For each type of cell assayed the mean and standard deviation of the invasion frequencies were calculated.

Regardless of which of the two types of substrates are employed, the invasion substrates of the present invention are easy to prepare and give rapid, highly consistent results with a variety of cells, including but not limited to fibroblasts and keratinocytes.

While not limited to any mechanism, it is believed that cells exposed to invasion-inducing agents in this manner are potentially rendered capable of invading the substrate. Again, while not limited to any mechanism, it is believed that the invasion inducing agent comprising the sequence PHSRN binds to the $\alpha 5\beta 1$ receptor on the cell and thereby induces invasion of the substrate. In this regard, the present invention provides a method of treating

cells comprising: a) providing i) cells expressing the $\alpha 5\beta 1$ receptor, ii) a fibronectin-free substrate, and iii) one or more invasion-inducing agents; b) culturing said cells (or at least maintaing the cells briefly) in serum-free culture media on said substrate in the presence of said invasion-inducing agents; and d) measuring the extent of cell invasion of said substrate. In one embodiment, the cells are human fibroblasts.

B. Compositions and Methods for the Treatment of Patients with Wounds

It is not intended that the present invention be limited by the nature of the wound healing promoting agent. Such agents can be identified functionally by simply testing them in the above-described *in vitro* assays. The extent of invasion of fibroblasts (or other suitable cells) with such agents is predictive of *in vivo* efficacy. Thus, the present invention contemplates *in vivo* treatment with "invasion-inducing agents," *i.e.* those agents which have the capability of causing invasion of cells (such as fibroblasts and keratinocytes) in the above-described *in vitro* assays.

1. Peptide Derivatives

In one embodiment, the invasion-inducing agent comprises a peptide derived from fibronectin. In a preferred embodiment, said peptide comprises the sequence PHSRN. Of course, the peptide may be larger than five amino acids; indeed, the peptide fragment of fibronectin may contain hundreds of additional residues (e.g. five hundred amino acids). One such larger peptide is set forth in U.S. Patent 5,492,890 (hereby incorporated by reference). In one embodiment, the PHSRN-containing peptide is less than one hundred amino acids in length and lacks the RGD sequence characteristic of fibronectin. A variety of PHSRN-containing peptides are contemplated, including the PHSRN peptide itself and related peptides where additional amino acids are added to the carboxy terminus, including (but not limited to) peptides comprising the sequence: 1) PHSRN, 2) PHSRNS, 3) PHSRNSI, 4) PHSRNSIT, 5) PHSRNSITL, 6) PHSRNSITLT, 7) PHSRNSITLTN, 8) PHSRNSITLTNL, 9) PHSRNSITLTNLT, 10) PHSRNSITLTNLTP, and 11) PHSRNSITLTNLTPG. Alternatively, PHSRN-containing peptides are contemplated where amino acids are added to the amino terminus, including (but not limited to) peptides comprising the sequence: 1) PEHFSGRPREDRVPHSRN, 2) EHFSGRPREDRVPHSRN, 3) HFSGRPREDRVPHSRN, 4) FSGRPREDRVPHSRN, 5) SGRPREDRVPHSRN, 6) GRPREDRVPHSRN,

7) RPREDRVPHSRN, 8) PREDRVPHSRN, 9) REDRVPHSRN, 10) EDRVPHSRN, 11) DRVPHSRN, 12) RVPHSRN, and 13) VPHSRN. Finally, the present invention contemplates PHSRN-containing peptides where amino acids are added to both the amino and carboxy termini, including (but not limited to) peptides comprising the sequence PEHFSGRPREDRVPHSRNSITLTNLTPG, as well as peptides comprising portions or fragments of the PHSRN-containing sequence PEHFSGRPREDRVPHSRNSITLTNLTPG.

Peptides containing variations on the PHSRN motif are contemplated. For example, the present invention also contemplates PPSRN-containing peptides for use in the above-named assays. Such peptides may vary in length in the manner described above for PHSRN-containing peptides. Alternatively, PPSRN may be used as a peptide of five amino acids.

Similarly, peptides comprising the sequence -HHSRN-, -HPSRN-, -PHTRN-, -HHTRN-, -HPTRN-, -PHSNN-, -HHSNN-, -HPSNN-, -PHTNN-, -HHTNN-, -HPTNN-, -PHSKN-, -HHSKN-, -HPSKN-, -PHTKN-, -HHTKN-, -HPTKN-, -PHSRR-, -HHSRR-, -HPSRR-, -PHTRR-, -HHTRR-, -HPTRR-, -PHSNR-, -HHSNR-, -HPSNR-, -PHTNR-, -HHTNR-, -HPTNR-, -PHSKR-, -HHSKR-, -HPSKR-, -PHTKR-, -HHTKR-, -HPTKR-, -PHSRK-, -HHSRK-, -HPSRK-, -PHTRK-, -HHTRK-, -HPTRK-, -PHSNK-, -HHSNK-, -HPSNK-, -PHTNK-, -HHTNK-, -HPTNK-, -PHSKK-, -HHSKK-, -HPSKK-, -PHTKK-, -HHTKK-, or -HPTKK- are contemplated by the present invention. Such peptides can be used as five amino acid peptides or can be part of a longer peptide (in the manner set forth above for PHSRN-containing peptides).

As noted above, the present invention contemplates peptides that are protease resistant. In one embodiment, such protease-resistant peptides are peptides comprising protecting groups. In a preferred embodiment, the present invention contemplates a peptide containing the sequence PHSRN (or a variation as outlined above) that is protected from exoproteinase degradation by N-terminal acetylation ("Ac") and C-terminal amidation. The Ac-XPHSRNX-NH_2 peptide (which may or may not have additional amino acids, as represented by X; the number of additional amino acids may vary from between 0 and 100, or more) is useful for *in vivo* administration because of its resistance to proteolysis.

In another embodiment, the present invention also contemplates peptides protected from endoprotease degradation by the substitution of L-amino acids in said peptides with their corresponding D-isomers. It is not intended that the present invention be limited to particular amino acids and particular D-isomers. This embodiment is feasible for all amino acids, except glycine; that is to say, it is feasible for all amino acids that have two stereoisomeric forms.

By convention these mirror-image structures are called the D and L forms of the amino acid. These forms cannot be interconverted without breaking a chemical bond. With rare exceptions, only the L forms of amino acids are found in naturally occurring proteins. In one embodiment, the present invention contemplates PHS(dR)N-containing peptides for wound healing

2. Mimetics

As noted above, compounds mimicking the necessary conformation for recognition and docking to the receptor binding to the peptides of the present invention are contemplated as within the scope of this invention. For example, mimetics of PHSRN peptides are contemplated for use in wound healing. The variety of designs for such mimetics are discussed above (albeit in the context of cancer). In one embodiment of the present invention, it is contemplated that the relevant peptide sequence is Pro-His-Ser-Arg-Asn or Pro-Pro-Ser-Arg-Asn. In one embodiment, the mimetics of the present invention are peptides having sequence homology to the above-described PHSRN-containing peptides (including, but not limited to, peptides in which L-amino acids are replaced by their D-isomers).

3. Formulations

It is not intended that the present invention be limited by the particular nature of the therapeutic preparation, so long as the preparation comprises an invasion-inducing agent. For example, such compositions can be provided together with physiologically tolerable liquid, gel or solid carriers, diluents, adjuvants and excipients.

These therapeutic preparations can be administered to mammals for veterinary use, such as with domestic animals, and clinical use in humans in a manner similar to other therapeutic agents. In general, the dosage required for therapeutic efficacy will vary according to the type of use and mode of administration, as well as the particularized requirements of individual hosts.

Compositions for wound healing are typically prepared as liquid solutions or suspensions, or in solid forms. Formulations for wound healing usually will include such normally employed additives such as binders, fillers, carriers, preservatives, stabilizing agents, emulsifiers, buffers and excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules.

sustained release formulations, or powders, and typically contain 1%-95% of active ingredient, preferably 2%-70%.

The compositions are also prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared.

The invasion-inducing agents of the present invention are often mixed with diluents or excipients which are physiological tolerable and compatible. Suitable diluents and excipients are, for example, water, saline, dextrose, glycerol, or the like, and combinations thereof. In addition, if desired the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, stabilizing or pH buffering agents.

Additional formulations which are suitable for other modes of administration, such as topical administration, include salves, tinctures, creams, lotions, and, in some cases, suppositories. For salves and creams, traditional binders, carriers and excipients may include, for example, polyalkylene glycols or triglycerides.

As noted above, the present invention specifically contemplates the use of solid supports, including but not limited to dressings. In one embodiment, the formulation is applied to a dressing and the dressing is applied to a wound.

III. Anti-Coagulants

As noted above, the present invention contemplates using the peptides, peptide derivatives, and non-peptide mimetics of the present invention as anti-coagulants. It is not intended that the present invention be limited to the particular patient status associated with anti-coagulant therapy. In one embodiment, the present invention contemplates treating or preventing cardiovasular disease.

Cardiovascular disease is a broad term encompassing many pathologies of the heart and vascular system, including hypertension, stroke, aneurysm, angina, myocardial infarction, and Raynaud's disease. During 1990, cardiovascular disease caused about 43% of the deaths -- more than 900,000 people -- in the United States. Thus, the number of deaths from cardiovascular disease was nearly as high as the number of deaths from all other causes combined. [J.T. Shepherd, *et al.*, "Report of the Task Force on Vascular Medicine," Circulation 89(1):532-35 (1994)].

Cardiovascular disease is also a leading cause of morbidity. Both patients and their families suffer a great deal from the effects of cardiovascular disease. Furthermore, there is a

tremendous economic impact associated with such illness. Both the high incidence and the often-severe manifestations of cardiovascular disease necessitate that a large portion of health care workers' time be devoted to the care of patients suffering from the disease state. Moreover, sufferers of cardiovascular disease lose countless numbers of productive hours each year due to their illness. It is important to remember that cardiovascular disease affects many people besides the elderly or those having a familial predisposition. Indeed, the establishment of detailed guidelines directed solely to the evaluation of congenital cardiac problems in pre-adults illustrates that the young are not immune from cardiovascular disease. [D. Driscoll *et al.* "Guidelines for Evaluation and Management of Common Congenital Cardiac Problems in Infants, Children, and Adolescents," Circulation 90(4):2180-88 (1994)].

A number of anti-thrombotic agents are currently known which inhibit clot formation by preventing platelet integrins from binding fibrinogen or fibronectin. These anti-thrombotics, however, rely on competitive inhibition to prevent platelet integrins from binding to fibrinogen or fibronectin. In this manner, large doses of these agents are required to achieve the desired anti-thrombotic affect.

The present invention contemplates a more effective approach using PHSRN-antagonists such as PHSCN. While the precise mechanism need not be known to practice the invention it has been shown that the platelet integrin, αIIbβ3, also binds the PHSRN sequence of plasma fibronectin. Thus, instead of utilizing competitive inhibition, the PHSRN-antagonists may directly inhibit platelet integrins from binding fibronectin and aggregating. Specifically, the PHSCN peptide, or other PHSRN-antagonists, may directly inhibit early stages in clot formation by binding to the αIIbβ3 receptors on platelets. This prevents platelet integrins from binding fibronectin, a necessary part of platelet aggregation, thus inhibiting an integral step in the blood clotting cascade. In this manner, a comparatively small dose of the PHSCN peptide, or other PHSRN antagonist, is contemplated as effective anti-thrombotic agents.

IV. Adjuvants

In the past, adjuvants were believed to function as non-specific immune simulators and are frequently used to enhance the production of antibody. By providing a pool of emulsified antigen at an injection site, adjuvants were thought to allow the antigen to be released slowly over an extended period of time, thereby enhancing and prolonging the immune response. *See generally*, I. Roitt *et al.*, *Immunology* (section 8.9) (Gower Medical Publishing 1985).

Equal portions of a diluent such as saline, in which the antigen is dissolved or suspended, and an emulsifier-mineral oil mixture (which may or may not contain mycobacteria) are mixed until a stable water in oil emulsion forms. Emulsification should be thorough and is particularly important if the antigen is a soluble protein. The resulting emulsion is satisfactory if a drop placed on the surface of water will not spread.

The most commonly used adjuvants in animals are Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA). CFA is a suspension of *Mycobacterium butyricum* in a mixture of paraffin oil and emulsifying agent mannide monoleate. IFA is similar to CFA except that *M. butricum* has been omitted.

The addition of mycobacteria to these adjuvants (such as in CFA) further enhances the immune response to antigens, particularly soluble antigens. However, these bactoadjuvants are only used in the preparation of antigen-adjuvant emulsions used in immunological studies with laboratory animals; they are not intended for human use or therapeutic use. Accidental human injections with CFA have resulted in severe inflammatory reactions.

On the other hand, the present invention contemplates adjuvants which can be safely administered in humans. The present invention contemplates adjuvants comprising a mixture of antigen and the fibronectin-derived peptides (as well as derivatives and non-peptide mimetics) of the present invention to immunize animals, including humans. In one embodiment, the present invention contemplates mixing antigen with a PHSRN-containing peptide (including but not limited to such a peptide which has been chemically modified to render it more resistant to proteases) and immunizing (whether subcutaneously, intramuscularly, intraperatoneally, etc.) with the mixture to generate antibody to the antigen. Thus, the present invention specifically contemplates compositions (including but not limited to vaccines) comprising antigen (such as antigens from pathogens, said pathogens including but not limited to bacterial and viral pathogens) and the fibronectin-derived peptides (such as a PHSRN-containing peptide) of the present invention, such compositions being useful to generate a specific immune response. While not limited to any mechanism by which the peptides of the present invention serve as adjuvants, it is believed that the peptides (and derivatives and non-peptide mimetics) of the present invention recruit immune cells to the site of antigen injection.

V. Gene Therapy

The present invention relates to the *in vivo* delivery of exogenous nucleic acids to cells, including but not limited to, the cells of multicellular organisms. The basis approach to delivery is known in the art. For example, Wu *et al.*, U.S. Patent 5,166,320 (hereby incorporated by reference), discloses tissue-specific delivery of DNA using a conjugate of a polynucleic acid binding agent (such as polylysine, polyarginine, polyornithine, histone, avidin, or protamine) and a tissue receptor-specific protein ligand. For targeting liver cells, Wu suggests asialoglycoprotein (galactose-terminal) ligands. In another example, Wagner *et al.*, *Proc. Natl. Acad. Sci.*, 88:4255-4259 (1991) and U.S. Patent No. 5,354,844 (hereby incorporated by reference) disclose complexing a transferrin-polylysine conjugate with DNA for delivering DNA to cells via receptor mediated endocytosis. Wagner, *et al.*, teach that it is important that there be sufficient polycation in the mixture to ensure compaction of plasmid DNA into toroidal structures of 80-100 nm diameter, which, they speculate, facilitate the endocytic event.

The present invention contemplates enhancing gene therapy using the fibronectin-derived peptides of the present invention. While the present invention is not limited to any mechanism by which gene therapy is thereby enhanced, it is believed that such peptides enhance angiogenesis (i.e. vascularization of the site) and/or recruit cells to the site of transfection, thereby permitting the introduction of nucleic acid into the recruited cells. The present invention contemplates using a mixture comprising nucleic acid (whether DNA or RNA, or nucleic acid containing nucleotide analogues) and the fibronectin-derived peptides (as well as derivatives and non-peptide mimetics) of the present invention to introduce nucleic acid into the cells of animals, including humans. In one embodiment, the present invention contemplates mixing nucleic acid encoding a gene of interest (and preferably a gene encoding a protein having beneficial properties to the receiving host) with a PHSRN-containing peptide (including but not limited to such a peptide which has been chemically modified to render it more resistant to proteases) and transfecting cells (whether those cells are liver cells, muscle cells, nerve cells, etc.) with the mixture (whether *in vivo* or *in vitro*) to introduce said nucleic acid into said cells. In one embodiment, the nucleic acid is contained within a vector and the nucleic acid is introduced under conditions such that said gene of interest is expressed. Thus, the present invention specifically contemplates compositions comprising nucleic acid (such as nucleic acid encoding a protein of interest, said protein of interest including but not limited to

proteins having a beneficial impact) and the fibronectin-derived peptides (such as a PHSRN-containing peptide) of the present invention.

It is not intended that the present invention be limited by the type of gene therapy employed. In a preferred embodiment, the nucleic acid comprises an expressible gene which is functional in the target cell. For example, the gene may encode coagulation factors, (such as Factor IX), enzymes involved in specific metabolic defects, (such as urea cycle enzymes, especially ornithine transcarbamylase, argininosuccinate synthase, and carbamyl phosphate synthase); receptors, (*e.g.*, LDL receptor); toxins; thymidine kinase to ablate specific cells or tissues; ion channels (*e.g.*, chloride channel of cystic fibrosis); membrane transporters (*e.g.*, glucose transporter); and cytoskeletal proteins, (*e.g.*, dystrophin). In one embodiment, the gene therapy addresses restenosis.

The gene may be of synthetic, cDNA or genomic origin, or a combination thereof. The gene may be one which occurs in nature, a non-naturally occurring gene which nonetheless encodes a naturally occurring polypeptide, or a gene which encodes a recognizable mutant of such a polypeptide. It may also encode an mRNA which will be "antisense" to a DNA found or an mRNA normally transcribed in the host cell, but which antisense RNA is not itself translatable into a functional protein.

For the gene to be expressible, the coding sequence must be operably linked to a promoter sequence functional in the target cell. Two DNA sequences (such as a promoter region sequence and a coding sequence) are said to be operably linked if the nature of the linkage between the two DNA sequences does not result in the introduction of a frame-shift mutation in the region sequence to direct the transcription of the desired gene sequence, or interfere with the ability of the gene sequence to be transcribed by the promoter region sequence. A promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence. In order to be "operably linked" it is not necessary that two sequences be immediately adjacent to one another. A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a mRNA if it contains nucleotide sequences which contain transcriptional regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the RNA. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism, but in general include a promoter which directs the initiation of RNA transcription. Such regions may include those 5'-non-coding sequences involved with initiation of transcription such as the TATA box.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); μ (micron); M (Molar); μM (micromolar); mM (millimolar); N (Normal); mol (moles); mmol (millimoles); μmol (micromoles); nmol (nanomoles); g (grams); mg (milligrams); μg (micrograms); ng (nanograms); L (liters); ml (milliliters); μl (microliters); cm (centimeters); mm (millimeters); μm (micrometers); nM (nanomolar);°C (degrees Centigrade); mAb (monoclonal antibody); MW (molecular weight); PBS (phophate buffered saline); U (units); d(days).

In some of the examples below, wounds are created in animals. Briefly, in one approach, experimental wounds were created in animals which were pre-anesthetized by inhalation of metofane and intradermal injection of lidocane. The hair on the backs of the animals was clipped and the skin was disinfected with 70% ethanol. A piece of skin was then removed from the disinfected site with a 4 mm punch biopsy.

EXAMPLE 1

Production Of Fibronectin-Free Substrates

This example describes a purification approach for removal of plasma fibronectin (and/or cellular fibronectin) from a substrate (Matrigel). In this example, removal was attempted by affinity chromatography over Gelatin-Sepharose (a technique which can be used to remove plasma fibronectin from fetal calf serum).

The Gelatin-Sepharose beads were obtained from Pharmacia (Catalog# 17-0956-01). Two Kontes columns were set up with about 2 mls of Gelatin-Sepharose beads at 4 C to prevent gelling of the Matrigel. The columns were then rinsed with about 10 column volumes of PBS to remove the preservative from the beads. The columns were drained to the top of the beads; then Matrigel was carefully added to the column. Once the Matrigel had entered the column, PBS was added to the top of the column. The Matrigel which was passed over the first column was collected and passed over the second column. The fibronectin-depleted Matrigel collected from the second column was plated on 48-well plates (150 μl/well), sterilized under a UV light for 10 minutes and incubated at 37 C overnight. The Matrigel treated in this manner failed to form a gel at 37 C.

EXAMPLE 2

Production Of Fibronectin-Free Substrates

This example describes a purification approach for removal of plasma fibronectin (and/or cellular fibronectin) from a substrate (Matrigel). In this example, removal was attempted by successive panning on gelatin. Eight wells of 24-well plate were coated with a 2% gelatin solution (the gelatin was obtained from Becton Dickinson Labware, Catalog #11868). The wells were filled with the gelatin solution which had been heated to 50 C and incubated for 3 minutes. Then the solution was removed and the wells were allowed to air dry. Following drying, the wells were thoroughly rinsed with ddH2O followed by two rinses with PBS. The plates were again allowed to dry; thereafter they were stored at -20 C until use. Matrigel was thawed on ice and then added to one of the wells of a gelatin-coated plate (between 800 µl and 1 ml of Matrigel was added to a well of a 24-well plate). The plate was placed in a bucket of ice in a 4 C room on an orbital shaker where the Matrigel was incubated in the well for two hours (although overnight incubation can be used). Following the incubation, the Matrigel was moved from the first well to a second well and then incubated for two hours under the same conditions. This process was repeated until the Matrigel had been incubated on all eight wells of the gelatin-coated plate.

Following the depletion of the Matrigel, it was collected in Eppendorf tubes. It was then plated on a 48-well plate 150 µl/well), sterilized under a UV light for 10 minutes and incubated at 37 C overnight. The Matrigel formed as gel and the following day, cells were added to each well.

EXAMPLE 3

Production Of Fibronectin-Free Substrates

This example describes a purification approach for removal of plasma fibronectin (and/or cellular fibronectin) from a substrate (Matrigel). In this example, removal was attempted by gelatin panning followed by antibody panning.

Anti-fibronectin antibody-coated wells: Wells of a 24-well plate were coated with an anti-fibronectin antibody. A mouse monoclonal antibody to human fibronectin was obtained from Oncogene Science (Catalog #CP13). Each well was incubated with 1 ml of antibody at a concentration of 30 µl/ml for 2 hours at room temperature. Each well was then incubated

with a solution of 3% BSA in PBS for 2 hours at room temperature. Following the two incubation periods, the wells were thoroughly washed with PBS and stored at -20 C until use.

Depleting Matrigel of Fibronectin: Matrigel was panned over eight gelatin-coated wells (as described above in Example 2) to remove most of the fibronectin and its fragments. Thereafter, the Matrigel was placed in the antibody-coated wells to remove any remaining fragments of fibronectin which contain the cell-binding domain but not the gelatin-binding domain. The Matrigel was incubated in an ice bucket on an orbital shaker at 4 C for 2 hours. Once the Matrigel was depleted, it was collected in Eppendorf tubes. The firbonectin-depleted Matrigel was plated on a 48-well plate (150 µl/well), sterilized under a UV light for 10 minutes and incubated at 37 C overnight. The Matrigel formed a gel and the following day, cells were added to the wells.

EXAMPLE 4

Inducing Invasive Behavior Of Tumor Cells

In this example, the role of plasma fibronectin in inducing the invasive behaviors of metastatic breast and prostate cancer cells is demonstrated. Human breast carcinoma cell lines SUM 52 PE and SUM 44 PE were originally cultured from the pleural effusions of patients with metastatic breast cancer; and SUM 102 was cultured from a primary, microinvasive breast carcinoma (Ethier, S.P., Mahack, M.L., Gullick, W.J., Frank, T.S., and Weber, B.L. Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media. Cancer Res. 53: 627-635). The DU 145 metastatic human prostate cancer cell line was originally cultured from a brain metastasis (Stone, K.R., Mickey, D.D., Wunderli, H., Mickey, G.H., Paulsen, D.F. (1978) Isolation of a human prostate carcinoma cell line (DU 145), Int. J. Cancer 21: 274-281. These cell lines can all be maintained for at least 24 hours in serum-free conditions; thus they are ideal for use in serum-free invasion assays on SU-ECM.

Adult *Strongylocentrotus purpuratus* sea urchins were obtained from Pacific BioMarine, and their embryos were cultured to the early pluteus stage in artificial sea water at 15°C. SU-ECM were prepared from them by treatment with nonionic detergent and strerilized by dilution in the appropriate media.

Cells were harvested by rinsing in Hanks' balanced salt solution, followed by brief treatment with 0.25% trypsin, 0.02% EDTA, and pelleting and resuspension in the appropriate

medium with or without 5% FCS at a density of about 50,000 cells per ml. When appropriate, purified bovine plasma fibronectin (Sigma), purified 120 kDa chymotryptic fragment (Gibco BRL), PHSRN or PHSCN peptides (synthesized at the Biomedical Research Core Facilities of the University of Michigan), or GRGDSP or GRGESP peptides (Gibco BRL) were added to the resuspended cells prior to placement of the cells on SU-ECM. In each well of a plate used for an invasion assay, SU-ECM were placed in 0.5 ml of the appropriate medium, and 0.5ml of the resuspended cells dropped on their exterior surfaces. Invasion assays were incubated 1 to 16 hours prior to assay. If some circumstances, invasion assays were fixed in phosphate-buffered saline (PBS) with 2% formaldehyde for 5 minutes at room temperature, then rinsed into PBS.

Invasion assays were coded and scored blindly by microscopic examination under phase contrast at 200- and 400-fold magnification. Each cell contacting an SU-ECM was scored for its position relative to the exterior or interior surfaces. A cell was judged to have invaded if it was located on an interior surface below the focal plane passing through the upper surface of the SU-ECM, but above the focal plane passing through its lower surface. The minimum viability of the cells in each assay was always ascertained at the time of assay by determining the fraction of spread, adherent cells on the bottom of each well scored.

An invasion frequency is defined as the fraction of cells in contact with basement membranes which were located in their interiors at the time of assay. Thus, an invasion frequency of 1 denotes invasion by 100% of the cells in contact with basement membranes. Invasion frequencies were determined multiple times for each cell type assayed. For each type of cell assayed the mean and standard deviation of the invasion frequencies were calculated.

The invasion-inducing sequences of plasma fibronectin were mapped to a peptide sequence 5 amino acids long, the PHSRN peptide, for both metastatic breast and prostate cancer cells. Since the PHSRN sequence is present in plasma fibronectin, a significant component of serum, eliciting the regulatory role of this sequence was only possible because of the availability of a serum-free *in vitro* invasion substrate. It should be noted that neonatal, human fibroblasts are also induced with the PHSRN peptide to invade serum-free SU-ECM. Although fibroblasts do not invade SU-ECM in the presence of serum, the 120 kDa fragment of plasma fibronectin containing the PHSRN sequence can induce fibroblast invasion equally well in the presence of serum or in its absence.

When taken together, the results of experiments showing that the PHSRN sequence of plasma fibronectin induces the invasive behaviors of both metastatic breast and prostate cancer cells, as well as that of normal fibroblasts suggest the intriguing possibility that the invasive behavior associated with tumor cell metastasis may result from defects in the regulation of the normal invasive behaviors associated with wound healing.

EXAMPLE 5

Testing Tumor Cells On Fibronectin-Depleted Substrates

This example describes an approach to test cancer cells *in vitro* on substrates with and without invasion-inducing agents. The depleted preparation of Matrigel (see Example 2, above) and untreated Matrigel were used to test DU-145 metastatic prostate cancer cells. When plated on the depleted medium, the cancer cells failed to invade the matrix (see Figure 2). Indeed, it was evident that these cells were sitting on the surface of the depleted Matrigel because the Matrigel surface was slightly tilted; this was visible through the microscope as a gradual progressive, uniform change in the focal plane for the monolayer of DU-145 cells.

The addition of 0.5 µl/ml of the PHSRN peptide to the depleted Matrigel was sufficient to restore the full DU-145 invasiveness (see Figure 3). Clearly, gelatin panning removes fibronectin such that cancer cells are unable to invade. Since the addition of PHSRN peptide in solution fully restores the DU-145 invasive phenotype, blocking the effect of PHSRN is an effective strategy for therapeutic intervention in tumor cell invasion and metastasis.

EXAMPLE 6

Improving Gelatin Depletion As Measured By Fibroblast Invasiveness

In this example, normal, neonatal fibroblasts were tested on the depleted Matrigel material prepared according to Example 3 above (*i.e.*, antibody depletion). As shown in Figure 4, panning with an antibody after gelatin depletion improved the method for removal, as measured by the reduced invasiveness of fibroblasts. On the other hand, invasiveness of the fibroblasts could be induced by the addition of the PHSRN peptide.

The success of antibody panning suggests the feasibility of removing other components by the antibody panning methods. Other serum components, such as thrombospondin, growth

factors and cytokines are contemplated by the present invention for removal by the appropriate (commercially available) antibody.

EXAMPLE 7

Conjugation Of PHSRN-Containing Peptides

In this example, the preparation of a peptide conjugate is described. The synthetic peptide NH_2 - PHSRNC can be prepared commercially (*e.g.*, Multiple Peptide Systems, San Diego, CA). The cysteine is added to facilitate conjugation to other proteins.

In order to prepare a protein for conjugation (*e.g.*, BSA), it is dissolved in buffer (*e.g.*, 0.01 M $NaPO_4$, pH 7.0) to a final concentration of approximately 20 mg/ml. At the same time n-maleimidobenzoyl-N-hydroxysuccinimide ester ("MBS" available from Pierce) is dissolved in N,N-dimethyl formamide to a concentration of 5 mg/ml. The MBS solution, 0.51 ml, is added to 3.25 ml of the protein solution and incubated for 30 minutes at room temperature with stirring every 5 minutes. The MBS-activated protein is then purified by chromatography on a Bio-Gel P-10 column (Bio-Rad; 40 ml bed volume) equilibrated with 50 mM $NaPO_4$, pH 7.0 buffer. Peak fractions are pooled (6.0 ml).

The above-described cysteine-modified peptide (20 mg) is added to the activated protein mixture, stirred until the peptide is dissolved and incubated 3 hours at room temperature. Within 20 minutes, the reaction mixture becomes cloudy and precipitates form. After 3 hours, the reaction mixture is centrifuged at 10,000 x g for 10 min and the supernatant analyzed for protein content. The conjugate precipitate is washed three times with PBS and stored at 4°C.

From the above, it should be clear that the present invention provides a method of testing a wide variety of tumor types, and in particular identifying invasive tumors. With a means of identifying such tumors (now provided by the present invention) and distinguishing such tumors from non-invasive cancer, the physician is able to change and/or optimize therapy. Importantly, the antagonists of the present invention (and other drugs developed by use of the screening assay of the present invention) will provide treatment directed an invasive cells (and therefore associated with minimal host toxicity).

EXAMPLE 8

Inhibiting Invasion Of Human Breast Cancer Cells

In this example, the role of the PHSCN peptide in inhibiting the invasive behavior of metastatic breast cancer cells is demonstrated. The method of Example 4 is employed, with the addition of varying concentrations of the PHSCN peptide.

Example 4 indicates that SUM-52 cells (in medium with 5% fecal calf serum) are induced to invade the SU-ECM substrate in the presence of serum fibronectin or just the PHSRN sequence of fibronectin. Thus, the procedure in Example 4 provides a method for determining the inhibitory potential of the PHSCN peptide by comparing the number of cell invasions in the presence of the PHSCN peptide, with the number of cell invasions in the absence of the PHSCN peptide.

The results of adding varying concentrations of the PHSCN peptide to serum-induced metastatic SUM-52 PE breast cancer cells are presented in Figure 5A. The logs of the PHSCN peptide concentrations in ng per ml are plotted on the X axis. The percentages of invaded SUM 52 PE cells relative to the percentage invaded in the absence of the PHSCN peptide are plotted on the Y axis. Mean invasion percentages are shown with their first standard deviations. Clearly, the PHSCN peptide exhibits a substantial inhibitory affect on these cells, even at relatively low concentrations. The PHSCN peptide's inhibitory affect is further demonstrated by the fact that relatively high concentrations cause complete inhibition.

The results of adding varying concentrations of the PHSCN peptide to PHSRN-induced invasion of both metastatic SUM-52 PE breast cancer cells (in serum free media) and normal human mammary epithelial cells (in 10% FCS), are presented in Figure 5B. All invasion assays were carried out in 100 ng per ml of the PHSRN peptide to induce invasion. Again, the PHSCN peptide exhibits a substantial inhibitory affect on both cell lines at low concentrations, and almost complete inhibition at higher concentrations.

This example demonstrates the PHSCN peptide is an effective inhibitor of human breast cancer cell invasion. In this manner, the PHSCN peptide, or related sequences, are likely to provide effective therapy for human breast cancer by preventing the lethal affects of tumor cell metastasis.

EXAMPLE 9

Inhibiting Invasion Of Human Prostate Cancer Cells

In this example, the role of the PHSCN peptide in inhibiting the invasive behavior of metastatic prostate cancer cells is demonstrated. The method of Example 4 is employed, with the addition of varying concentrations of the PHSCN peptide.

Example 4 indicates that DU-145 cells are induced to invade the SU-ECM substrate in the presence of serum fibronectin or just the PHSRN sequence of fibronectin. Thus, the procedure in Example 4 provides a method for determining the inhibitory potential of the PHSCN peptide by comparing the number of cell invasions in the presence of the PHSCN peptide. with the number of cell invasions in the absence of the PHSCN peptide.

The results of adding varying concentrations of the PHSCN peptide to serum-induced invasion of metastatic DU-145 prostate cancer cells (in 10% serum) are presented in Figure 6A. The logs of the PHSCN concentrations are plotted on the X axis. The percentages of invaded DU-145 cells relative to the percentage invaded in the absence of the PHSCN peptide are plotted on the Y axis. Mean invasion percentages are shown with their first standard deviations. Clearly, the PHSCN peptide exhibits a substantial inhibitory affect on these cells, even at relatively low concentrations. The PHSCN peptide's inhibitory affect is further demonstrated by the fact that relatively high concentrations cause complete inhibition.

The results of adding varying concentrations of the PHSCN peptide to PHSRN-induced metastatic DU-145 prostate cancer cells (in serum-free medium) or to normal human prostate epithelial cells (in 10% FCS), are presented in Figure 6B. All invasion assays were carried out in 100 ng per ml of the PHSRN peptide to induce invasion. Again, the results show that the PHSCN peptide exhibits a substantial inhibitory affect on both cell lines at low concentrations, and almost complete inhibition at higher concentrations.

This example demonstrates the PHSCN peptide is an effective inhibitor of human prostate cancer cell invasion. In this manner, the PHSCN peptide may provide an effective therapy for human prostate cancer by preventing the lethal affects of tumor cell metastasis.

EXAMPLE 10

Inhibiting Invasion Of Rat Prostate Cancer Cells

In this example, the role of the PHSCN peptide in inhibiting the invasive behavior of rat metastatic prostate carcinoma MatLyLu (MLL) cells is demonstrated (see Example 4 for the general procedure employed). The result of adding 1 microgram per ml of the PHSCN peptide to serum-induced MLL cells causes complete inhibition of invasion (see Figure 7A).

The result of adding a varying concentration of the PHSCN peptide to PHSRN-induced MLL cells in serum free media is shown in Figure 7B, where 100 ng per ml of PHSRN was used to induce invasion. Figure 7B indicates that the PHSCN peptide exhibits a substantial inhibitory affect even at low concentrations, and almost complete inhibition at higher concentrations. This example demonstrates invasion of rat prostate cancer cells is inhibited in the same manner as human breast cancer cells (see Example 8) and human prostate cancer cells (see Example 9).

EXAMPLE 11

Inhibiting Invasion Of Rat Prostate Cancer Cells

In this example, the role of a homo-cysteine containing peptide (*i.e.*, PHS(hC)N) in inhibiting the invasive behavior of rat metastatic prostate carcinoma MatLyLu (MLL) cells is demonstrated. The procedure described in Example 10, was employed using SU-ECM substrates in 10% FCS and PHS(hC)N instead of PHSCN. The result of adding varying concentrations of the PHS(hC)N peptide to serum-induced MLL cells indicates this peptide also has an inhibitory affect on cell invasion (see Figure 8). As with the PHSCN peptide, the PHS(hC)N peptide substantially inhibits invasion at lower concentrations, and completely inhibits invasion at higher concentrations. This example demonstrates that the PHS(hC)N peptide has a similar inhibitory affect as the PHSCN peptide.

EXAMPLE 12

Inhibiting Growth And Metastasis Of Prostate Cancer Tumors *In Vivo*

In this example, the role of the PHSCN peptide in inhibiting the growth and metastasis of prostate cancer tumors *in vivo* is demonstrated. In the first part of this example, four

Copenhagen rats were injected with 500,000 MatLyLu (MLL) cells subcutaneously in the thigh. Two of these rats also received 1 mg of the PHSCN peptide along with the injected MLL cells, and thereafter received 1 mg of the PHSCN peptide injected in their tail vein three time per week for two weeks. The other two injected rats were left untreated. Tumor sizes were measured with calipers on day 14, and the tumors in the untreated rats were removed. The results depicted in Figure 9A, clearly demonstrate that the PHSCN peptide significantly slows the growth of injected MLL tumors in rats. It is possible that the ability of the PHSCN peptide to slow tumor growth is due to its inhibition of tumor invasion by normal endothelial cells, an anti-angiogenic effect.

Two weeks after the size of the tumors were measured, the rats were sacrificed and the mean number of lung metastases was determined at 10-fold magnification. The mean number of lung metastases in the untreated mice (MLL only) was nearly 35 in spite of the fact that the initial prostate tumors had been removed when their size was measured. The mean number of lung metastases in the treated mice (MLL + PHSCN) was less than 5, even though the initial prostate tumors were never removed because they were too small. This striking difference in mean number of metastases, depicted in Figure 9B. indicates that the PHSCN peptide significantly inhibits tumor cell metastasis in rats. In this manner, the PHSCN peptide provides effective *in vivo* therapy for cancer by preventing the lethal effects of tumor cell growth and metastasis.

EXAMPLE 13

Inhibiting Growth And Metastasis Of Prostate Cancer *In Vivo*

In this example, as in Example 12, the role of the PHSCN peptide in inhibiting the growth and metastasis of prostate cancer tumors *in vivo* is demonstrated. In the first part of this example, 20 Copenhagen rats were injected with 500,000 MatLyuLu (MLL) cells subcutaneously in the thigh. To more closely approximate a real clinical situation, PHSCN peptide treatment of 10 of these rats was initiated after 24 hours, instead of immediately. The 10 treated rats (MLL/PHSCN) received a total of 5 i.v. injections of 1 mg of the PHSCN peptide through the tail vein over two weeks. Tumor sizes were measured with calipers on day 14, and the tumors in the untreated rats were removed. Since the injected tumors in the MLL/PHSCN rats were still small, they were retained in the rats for another 7 to 9 days following the last PHSCN injection. At this time, their sizes were all greater than 2 cm, and

they were also removed. The result of the first part of this example, depicted in Figure 10A, clearly indicates that the PHSCN peptide, even when administered after the tumor cells have "seeded", substantially slows the growth of rat prostate cancer tumors.

The dramatic growth-inhibitory effect of the PHSCN peptide on MLL tumors may be due to their inhibition of the invasion of host endothelial cells into the tumor. Host endothelial cell invasion may be induced by the secretion of large amounts of proteinases from the tumors, and the resulting fragmentation of host plasma fibronectin. Fibronectin fragments have been shown to stimulate the migratory/invasive behaviors of normal mesenchymal and endothelial cells. This angiogenic process is believed to occur during normal wound healing. Thus, the ability of metastatic cells to be constitutively induced by intact plasma fibronectin to express proteinases and invade may play a central role both in tumor cell invasion and in tumor growth. In this manner, the PHSCN peptide is an effective chemotherapeutic to prevent the growth of tumors *in vivo*.

In the second part of this example, the MLL/PHSCN rats received 2 more i.v. doses of the PHSCN peptide prior to sacrifice. Ten days after the sizes of the injected primary tumors were determined, all the rats in the two groups (MLL only and MLL/PHSCN) were sacrificed, and the number of lung metastases was determined at 7.5-fold magnification. As can be seen in Figure 10B, there is a significant reduction in the mean numbers of lung metastases in the rats which received PHSCN treatment as compared to the untreated rats.

The 20 rats described in parts one and two of this example were also examined for metastatic tissues in their lymphatic systems. All of these metastases were dissected and weighed. Figure 10C plots the mean masses of intraperitoneal metastases (grams) for the two groups of 10 rats. As is clearly demonstrated, there is a significant reduction in the mean masses of lymphatic metastases in the rats which received PHSCN peptide treatment, as compared to the untreated rats. This may be due to the anti-angiogenic effect of the PHSCN peptide, as described in part one of this example. In this manner, the PHSCN peptide maybe an effective anti-metastatic, growth-inhibiting chemotherapeutic agent for use in the treatment of cancer.

EXAMPLE 14

Chemically Modified Peptides And Cancer Therapy

In this example, *in vitro* invasion of cancer cells into substrates is inhibited with peptides that have been chemically modified with protecting groups and peptides having

protecting groups as well as the modification wherein an L-amino acid has been replaced with the D-isomer. PHSCN peptide, blocked PHSCN peptide, and blocked PHSCN peptide with a D-cysteine instead of the L-isomer, were tested (Figure 11). The PHSCN peptide without protecting groups (*i.e.* "unblocked" PHSCN, open circles) completely inhibits the invasion of metastatic DU145 cells in medium with 10% serum at a concentration of 3 ng/ml. When PHSCN is protected from exoproteinase degradation by N-terminal acetylation and C-terminal amidation, the Ac-PHSCN-NH_2 peptide (closed circles) can completely inhibit the invasion of metastatic DU145 cells in medium with 10% serum at a concentration of approximately 1.3 ng/ml. Substitution of the D-isomer of cysteine (making Ac-PHS(dC)N-NH_2 for protection against endoproteolytic activity, striped circles) further increases the invasion-inhibitory activity of the peptide.

While not intending to limit the invention to any particular mechanism, it is believed that increases in activity may be due to proteinase resistance. Such proteases may be found in the serum or may be produced by the DU145 cells (i.e. both membrane-bound and secreted proteases). If proteinase resistance is the reason for the additional activity, this suggests that such modified peptides will have longer circulating half-lives upon administration to animals, including humans. This will permit lower dosages for therapy.

Alternatively, these changes may make the inhibitors interact with the PHSRN-binding pocket of the $\alpha5\beta1$ receptor more effectively. That is to say, the addition of groups on the ends of the peptide may have a positioning or steric effect that is beneficial for binding.

EXAMPLE 15

Structure/Function Relationships And Designing Non-peptide Mimetics

In this example, *in vitro* invasion of cancer cells into substrates is inhibited with non-peptide mimetics in comparison with chemically modified peptides (on a molar basis). The peptides of Example 14 (*i.e.* peptides chemically modified with protecting groups and peptides having protecting groups as well as the modification wherein an L-amino acid has been replaced with the D-isomer) were compared with the non-peptide mimetics DL-N-Acetylhomocysteinethiolactone, Thiobutyrolactone, and Mercaptopropionic acid, in order to investigate the structure activity relationship for invasion inhibition by interaction with the PHSRN-binding pocket of the $\alpha5\beta1$ fibronectin receptor (Figure 12)

The PHSCN peptide without protecting groups (*i.e.* "unblocked" PHSCN, open squares) completely inhibits the invasion of metastatic DU145 cells in medium with 10% serum at a concentration of approximately 2 μM. When PHSCN is protected from exoproteinase degradation by N-terminal acetylation and C-terminal amidation, the Ac-PHSCN-NH_2 peptide (striped squares) can completely inhibit the invasion of metastatic DU145 cells in medium with 10% serum at a concentration of approximately 0.06 μM. Substitution of the D-isomer of cysteine (making Ac-PHS(dC)N-NH_2 for protection against endoproteolytic activity. closed squares) further increases very substantially the invasion-inhibitory activity of the peptide.

The non-peptide mimetic, DL-N-Acetylhomocysteinethiolactone (closed circles) fully inhibits DU145 invasion at a concentration of 0.6 μM. Thus, is about 3-fold more active than the unblocked PHSCN peptide and about 10-fold less active than blocked PHSCN. Thiobutyrolactone (striped circles) fully inhibits DU145 invasion at a concentration of 30 μM. Thus, it is 15 times less active than the unblocked PHSCN peptide and about 500-fold less active than blocked PHSCN. It is also 50-fold less active than N-acetylhomocysteinethiolactone. Mercaptopropionic acid (open circls) did not reduce DU145 invasion by more than 20% at any of the concentrations tested. Thus, it is at least 100 times less active in invasion inhibition than thiobutyrolactone, and 1500 times less active than the unblocked PHSCN peptide.

The results of (Figure 12) can be examined in the context of shared structural motifs by comparing the compounds (Figure 13). The PHSCN peptide contains cycsteine and the sulfhydryl group of its cysteine has the potential to form a 6-membered ring by interacting with the carbonyl oxygen of its serine residue to chelate a divalent cation known to reside in the PHSRN-binding pocket of the α5β1 integrin fibronectin receptor. The chelation of this divalent cation may prevents its displacement by the arginine of the PHSRN sequence to activate invasion. This activity may explain how PHSCN functions as a competitive inhibitor of PHSRN-induced invasion. The results with the non-peptide mimetic DL-N-Acetylhomocysteinethiolactone suggest that the amino acids surrounding the cysteine in PHSCN may contribute a 10-fold increase in its invasion-inhibiting activity, presumably by increasing its association with the PHSRN-binding pocket of the α5β1 fibronectin receptor. N-Acetylhomocysteinethiolactone has the possibility of forming a 6-membered ring, very similar to that proposed for the PHSCN invasion-inhibitory peptide, by chelating a divalent cation between its electron-rich sulfhydryl group and its carbonyl oxygen or hydroxyl group

(after hydrolysis). It also has attached a nitrogen and a carbonyl group joined by a peptide linkage, which might resemble the peptide bond between the serine and histidine in the PHSCN peptide. These groups may contribute significantly to its invasion-inhibitory activity.

Thiobutyrolactone, like the homocysteinethiolactone described above, can potentially form a 6-membered ring by chelating a divalent cation between its electron-rich sulfhydryl group and its carbonyl oxygen or hydroxyl group (after hydrolysis). However, it lacks the attached peptide-linked nitrogen and carbonyl groups of the thiolactone. Thus, these attached groups may contribute a 50-fold increase in invasion inhibition.

Finally, Mercaptopropionic acid, unlike thiobutyrolactone, has the potential to form only a 5-membered ring upon interaction with the divalent cation. The poor results with this compound suggest that the presence of a 6-membered ring in the inhibitor after divalent cation chelation may increase invasion-inhibitory activity by 100-fold by stabilizing the presence of the divalent cation in the PHSRN-binding pocket. It is known that the divalent cation in this pocket is bound by 4 aspartic acids in the $\alpha 5$ chain of $\alpha 5\beta 1$. It is interesting that the inclusion of this metal ion between the electron-rich -SH and -O or -OH groups would lead to its chelation by 6 electron-rich moities instead of 4, a much more stable configuration. The ability of the PHSCN peptide, N-Acetylhomocysteinethiolactone, and thiobutyrolactone inhibitors to form 6-membered rings as they include the divalent cation in these 6 electron-rich motifs may contribute significantly to their invasion-inhibitory activities because 6-membered organic rings are energetically favored.

The presence of an NH-COOH linked to the 6-membered ring may increase invasion-inhibitory activity by another 50-fold by mimicing the peptide linkage of the serine and histidine. This suggests that this linkage interacts with the amino acids of the PHSRN-binding pocket in a significant way. The presence of the other amino acids in the PHSCN peptide may contribute another 10-fold increase in invasion-inhibitory activity by interacting with the amino acids of the PHSRN-binding pocket of the $\alpha 5\beta 1$ integrin fibronectin receptor.

EXAMPLE 16

Tests with a Simple Disulfide

In this example, a simple disulfide which is a known metal chelator was used in a test to inhibit serum-induced human cancer cell invasion. The commercially-available compound bis(diethylthio-carbamoyl) disulfide (known as "Antabuse") was employed. Antabuse

produces a sensitivity to alcohol. Figure 14 shows the results. The compound shows no significant impact on cancer cell invasion.

EXAMPLE 17

Effect of Serum on PHSRN on Induction of Human Fibroblast Invasion

In this example, the invasiveness of neonatal fibroblasts into an SU-ECM invasion substrate is considered. Experiments were performed under serum-free conditions, or in medium with 10% fetal calf serum (FCS). Neither serum-free medium nor medium containing serum supported fibroblast invasion. However, consistent with the induction of metalloproteinase gene transcription by the 120 kDa fragment of plasma fibronectin (pFn) containing the cell-binding domain, the 120 kDa fragment induced fibroblast invasion in the presence or in the absence of serum.

To insure the induction of invasion documented in these experiments was due to pFn sequences, and not to bound growth factors or cytokines, all of the fragments used were purified by electrophoresis on denaturing gels, followed by electoelution. Also, all fragments and sequences tested here present in solution at a molar concentration equivalent to that of plasma fibronectin in serum. The 120 kDa cell binding domain consists of modules 2 through 11. Modules 9 and 10 are bound by the $\alpha 5\beta 1$ receptor because module 9 contains the PHSRN sequence, while module 10 has the RGD sequence. Accordingly, the invasion-inducing activities of a gel-purified 39 kDa fragment containing modules 7-9 (and the PHSRN sequence) with a gel-purified 11.5 kDa fragment containing module 10 (and the RGD) sequence was considered. As can be seen in Figure 15, all of the invasion-inducing activity of the plasma fibronectin cell-binding domain appeared to map to the 39 kDa fragment bearing modules 7-9 and the PHSRN sequence. To test this observation rigorously, the PHSRN peptide, which was synthesized in a peptide synthesis CORE facility, and the GRGDS peptide, which was obtained commercially, were tested in the presence or in the absence of serum for their invasion-inducing activities. As shown in Figure 15, the PHSRN sequence contained all the invasion-stimulatory activity of the pFn cell-binding domain; and the RGD sequence had no detectable activity at the near-physiological concentrations used.

EXAMPLE 18

Dose-response Effect Between PHSRN Concentration and Fibroblast Invasion.

In this example, fibroblasts were induced to invade SU-ECM by concentrations of the PHSRN peptide ranging from 10 to 3000 ng per ml in the presence, or in the absence of serum. As can be seen from the dose response curves shown in Figure 16, the PHSRN peptide was able to induce fibroblast invasion in the presence of serum, which has been found to contain 40 to 80 micrograms per ml of intact plasma fibronectin, and in its absence in a similar log-linear fashion. D.F. Mosher "Physiology of Fibronectin" *Ann. Rev. Med.* 35:561 (1984).

These data suggest the metalloproteinase gene repressors produced by fibroblast $\alpha 4\beta 1$ and $\alpha 5\beta 1$binding of intact plasma fibronectin do not appear to bind with such high affinity that they stop PHSRN-mediated invasion induction in the presence of serum. P. Huhtala et al. "Cooperative Signaling by $\alpha 5\beta 1$ and $\alpha 4\beta 1$ Integrins Regulates Metalloproteinase Gene Expression in Fibroblasts Adhering to Fibronectin" *J. Cell Biol.* 129: 867 (1995). This observation is consistent with the fact that, although induced by fibronectin fragments, fibroblast invasion *in vivo* must occur in the presence of intact plasma fibronectin.

EXAMPLE 19

Induction of Keratinocyte Invasion by PHSRN in the Presence of Serum

In this example the induction of normal keratinocyte invasion by PHSRN peptide, in the presence of serum, is presented. Normal neonatal keratinocytes were tested for their ability to be induced to invade SU-ECM by the PHSRN peptide. It is notable that the profile of keratinocyte invasive induction into SU-ECM by PHSRN, presented in Figure 17, is similar to the profile of invasive induction of fibroblast presented in Example 18. These data present the maximal invasion percentages for keratinocytes at a level of about 20%. Treatment of the cells (e.g. trypsin treatment) and assay conditions (e.g. time or orientation) are likely to effect this level. In any event, it is preferred that measurements are taken in the linear range.

EXAMPLE 20

Invasiveness of Normal Human Mammary or Prostate Epithelial Cell in Response to Induction By PHSRN in a Serum Containing Environment

In this example, data is presented, Figure 18, demonstrating that PHSRN peptide also induces the invasive behaviors of human mammary or prostate epithelial cells. These experiments were conducted in a serum containing environment using SU-ECM as an invasion substrate. As with fibroblasts, immunostaining experiments showed that mammary and prostate epithelial cells express both the $\alpha5\beta1$ and the $\alpha4\beta1$fibronectin receptors (not shown); thus the ability of the $\alpha5\beta1$ receptor to bind the PHSRN sequence on fibronectin fragments lacking the $\alpha4\beta1$ binding site, which are generated in wounds may induce these epithelial cells to migrate into the provisional matrix or into its adjacent stroma to begin wound reepithelialization.

EXAMPLE 21

Invasiveness of Mouse Muscle Satellite Cells in Response to Induction by PHSRN in a Serum Containing Environment

In this example, the ability of the PHSRN peptide to induce the invasive behavior of a third major tissue type, muscle cells, was considered. Mouse muscle satellite cells, which function as stem cells for muscle *in vivo*, were obtained from the laboratory of Dr. K. Kurachi (Department of Human Genetics). These cells were placed on SU-ECM invasion substrates in 1 microgram per ml of PHSRN peptide in the presence or absence of serum. As shown in Figure 19, PHSRN induced the invasion of SU-ECM by muscle satellite cells. Since muscle satellite cells are normally located inside the basement membranes surrounding the muscle fibers, in direct contact with muscle cells, and since genetically engineered muscle cells have so far failed to cross the basement membranes separating them from the muscle fibers *in vivo*, it is interesting to speculate that treatment with the PHSRN invasion-inducing peptide may induce muscle satellite cell migration into muscle *in vivo*, where these cells might resume normal function.

EXAMPLE 22

In Vivo Effect of PHSRN

In this example, the effect of PHSRN on a dermal wound created on genetically obese, diabetic mice was considered. Figure 20 presents the data from 10 mice wounded with 4 mm biopsy punch through skin on the back. Just after wounding, the 5 treated mice receive 5 microliters of normal saline containing 2 micrograms PHSRN peptide in their wounds. The 5 untreated mice received 5 microliters of normal saline without PHSRN. Wound areas were measured on the days indicated by standard procedures in which a microscope slide is placed directly on the wound and its edges traced. The results show the rate of wound closure was accelerated in the PHRSN-treated group as compared to the untreated controls.

EXAMPLE 23

Comparative Wound Areas

In this example, the ratios of mean wound areas in PHSRN-treated and untreated wounds in normal and diabetic mice are considered. With respect to the diabetic mice, ten obese diabetic C57B16Ksdb/db mice received dermal wounds with a biopsy punch on day 0 according to standard methods. The wounds of 5 of these mice received 2 ug of the PHSRN peptide in 5µl normal saline without peptide. On the days indicated, the edges of all wounds were traced onto glass slides and the areas of the tracings determined in square mm. This is a standard method for wound area measurement in these mice. Six tracings of every wound were done on each day shown, and the mean wound areas determined.

With respect to non-diabetic littermates, eight non-diabetic C57B16Ks db/+ mice received duplicate dermal wounds with a biopsy punch on day 0 according to standard methods. 4 to 8 mm of unwounded skin separated each pair of wounds. One wound on each mouse received the PHSRN peptide as described above for the diabetic mice. The other wound received normal saline. On the days indicated, the edges of all wounds were traced onto glass slides for areas determination.

As shown in Figure 21, diabetic (db/db) and normal (db/+) mice both present ratios which fall to zero. This means the treated wounds all closed prior to the untreated ones. If the PHSRN had no effect, the ratios of the wound areas should remain at about 1. In the alternative, if the PHSRN peptide slowed wound healing with respect to untreated mice, the ratios should rise to infinity. Thus, a single application of the PHSRN peptide to the wound

shortly after wounding dramatically stimulated wound healing in both normal and diabetic mice.

It should also be noted that the rate of wound healing in the treated cohort of mice, relative to the untreated cohort, is promoted approximately equally in normal and diabetic mice through day four. This interval corresponds to the time of provisional matrix induction (which requires invasion by fibroblasts, Leukocytes, and blood vessels). It should be noted from these data that in the later stages of wound healing (after the first 4 days) the diabetic mice are less responsive than the normal mice. These data are consistent with the hypothesis that other late-occurring processes, which are PHSRN-independent, may still occur more slowly in diabetic mice as compared to their normal littermates.

Expanding upon the data presented in Figure 21, Figure 22 presents the percentages of completely closed wounds in PHSRN-treated and untreated diabetic mice during the 41 day period after wounding by the above described wounding methods. Again, the results show that a single application of the PHSRN peptide to the wound shortly after wounding stimulated wound healing in diabetic mice.

EXAMPLE 24

Inhibiting Blood Clotting

In this example, a blood clotting experiment was performed. The experiment was prompted by chance observations made during previous experiments in which the anti-tumorigenic and anti-metastatic activities of PHSCN were tested in rats. Since healthy rats, which received at total of 7 intravenous 1mg doses of the PHSCN peptide had no apparent ill effects, but also showed significantly increased blood clotting times, it appears that PHSCN or structurally related compounds may be effective, non-toxic anti-coagulants. It should also be noted that the 100 g rats used in previous experiments to assess the anti-tumorigenic or anti-metastatic activities of the PHSCN peptide would have had about volumes of total body fluids of about 50 to 75 ml, thus resulting in maximal PHSCN concentrations of 13 to 20 mg per ml in the blood. However, the PHSCN peptide concentrations in the peripheral blood are likely to have been less than the 20 microgram maximum because the difficulties in clotting were observed 24 hours after intravenous administration of the PHSCN peptide, during which time the concentration of the peptide may have decreased somewhat due to clearing and/or degradation.

The experiment was carried out in standard glass tubes (13 mm x 100 mm). A constant total volume of phosphate-buffered saline (30 microliters) was present in each tube. This liquid contained the following masses of the PHSCN peptide: 0 μg, 1 μg, 3 μg, 10 μg, or 30 μg. Exactly 1 ml of freshly-drawn venous blood was added to each tube and the clotting times determined by the methods described in Lee and White, "A clinical study of the coagulation times of blood," *Am. J. Med. Sci.* 145: 495 (1913) and modified by Tocantins and Kazal, *Blood Coagulation, Hemorrhage, and Thrombosis* (New York, Grune & Stratton, Inc.) (1964).

The percentage increase in whole blood clotting time using blood containing various concentrations of the PHSCN peptide was compared with blood without any peptide. The percentage increase in blood clotting time is plotted in Figure 23 on the Y axis and the log of the PHSRN concentration is shown on the X axis. The actual clotting time for the blood without any peptide was 12.5 minutes. The actual clotting time for the blood containing 10 μg/ml PHSCN was 17.0 minutes.

These data show that, at an easily obtainable PHSCN concentration in the peripheral blood, the clotting time was slowed by nearly 40%. Also, these is a clear optimal concentration for inhibition. The lack of linearity (or log-linearity) is very typical for blood clotting because it involves both platelet aggregation and multiple, inter-dependent regulatory and proteolytic events.

It is believed that further increases in clotting time would be achieved with chemically modified peptides, such as peptides containing protecting groups as well as peptides where one or more L-amino acids are substituted with their D-isomers.

From the above, it should be evident that the present invention provides methods and compositions for a variety of uses. First, it should be clear that the present invention provides an anticancer approach that is reliable for a wide variety of tumor types, and particularly suitable for invasive tumors. Importantly, the treatment is effective with minimal host toxicity.

Second, compositions and methods are described for enhancing and promoting wound healing. Invasion-inducing agents can be readily identified using the assays described above. Thereafter, such agents can be modified or derivatized and used therapeutically by application directly on wounds.

In addition, compositions and methods are described for anti-coagulants, adjuvants and enhancers for gene therapy.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Livant, Donna L.

(ii) TITLE OF INVENTION: Invasion-Inducing Agents and Invasion-Inhibitors for Use in Wound Healing and Cancer

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Medlen & Carroll, LLP
(B) STREET: 220 Montgomery Street, Suite 2200
(C) CITY: San Francisco
(D) STATE: California
(E) COUNTRY: United States of America
(F) ZIP: 94104

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Carroll, Peter G.
(B) REGISTRATION NUMBER: 32,827
(C) REFERENCE/DOCKET NUMBER: UM-03024

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (415) 705-8410
(B) TELEFAX: (415) 397-8338

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Pro His Ser Arg Asn
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Pro His Ser Cys Asn
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Cys His Ser Arg Asn
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Pro Cys Ser Arg Asn
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Pro His Cys Arg Asn
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Pro His Ser Arg Cys
1 5

CLAIMS

1. A composition, comprising a compound which inhibits the tumor invasion-promoting activity of an invasion-inducing peptide comprising the amino acid sequence PHSRN.

2. The composition of Claim 1, wherein said compound is a peptide derivative.

3. The composition of Claim 1, wherein said compound is an invasion-inhibiting peptide comprising the amino acid sequence X_1HSX_2N, wherein X_1 is either proline, histidine, an amino acid analogue or not an amino acid, and X_2 is an amino acid selected from the group consisting of the L-isomer of cysteine, the D-isomer of cysteine, homo-cysteine, histidine, and penicillamine.

4. The composition of Claim 3, wherein said invasion-inhibiting peptide comprises the amino acid sequence PHSCN.

5. The composition of Claim 3, wherein said invasion-inhibiting peptide contains additional amino acids added to the amino terminus, the carboxyl terminus, or both the amino and carboxyl termini.

6. The composition of Claim 5, wherein said invasion-inhibiting peptide is between four and five hundred amino acids in length.

7. The composition of Claim 5, wherein said invasion-inhibiting peptide's amino terminus is blocked with a protecting group.

8. The composition of Claim 5, wherein said invasion-inhibiting peptide's carboxyl terminus is blocked with a protecting group.

9. The composition of Claim 5, wherein said invasion-inhibiting peptide's amino terminus is blocked with an acetyl group, and said peptide's carboxyl terminus is blocked with an amide group.

10. The composition of Claim 3, wherein said invasion-inhibiting peptide is resistant to exoproteinases.

11. The composition of Claim 3, wherein said invasion-inhibiting peptide is resistant to endoproteinases.

12. The composition of Claim 1, wherein said compound is a non-peptide mimetic.

13. The composition of Claim 3, wherein said invasion-inhibiting peptide is suspended in serum-free media.

14. The composition of Claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12, wherein said compound is in a formulation suitable for administration to a human cancer patient.

15. The composition of Claim 1, further comprising blood. serum or plasma.

16. A method of treating cancer comprising:

(a) providing:

(i) a subject having cancer, and

(ii) the composition of Claim 14;

(b) administering said composition to said subject.

17. The method of Claim 16, wherein said composition is administered to said subject before or after the surgical removal of a tumor.

18. The method of Claim 16, wherein said composition is intravenously administered.

19. A composition, comprising a compound which promotes the invasion of cells into an invasion substrate.

20. The composition of Claim 19, wherein said compound is a peptide derivative.

21. The composition of Claim 19, wherein said compound comprises a peptide comprising the amino acid sequence X_1HSX_2N, wherein X_1 is either proline, histidine, an amino acid analogue or not an amino acid, and X_2 is an amino acid selected from the group consisting of the L-isomer of arginine and the D-isomer of arginine.

22. The composition of Claim 19, wherein said compound comprises a fibronectin-derived peptide comprising amino acids, wherein at least a portion of said peptide comprises the sequence PHSRN.

23. The composition of Claim 22, wherein said fibronectin-derived peptide has the amino acid sequence PHSRN.

24. The composition of Claim 22, wherein said fibronectin-dervied peptide comprises the amino acids PHSRN and additional amino acids added to the amino terminus.

25. The composition of Claim 22, wherein said fibronectin-dervied peptide comprises the amino acids PHSRN and additional amino acids added to the carboxy terminus.

26. The composition of Claim 22, wherein said fibronectin-dervied peptide comprises the amino acids PHSRN and additional amino acids added to both the amino and carboxy termini.

27. The composition of Claim 22, wherein said fibronectin-derived peptide peptide lacks the RGD motif.

28. The composition of Claim 22, wherein said fibronectin-derived peptide lacks the motif which binds the $\alpha 5\beta 1$ receptor.

29. The composition of Claims 21, 22, 24, 25, 26, 27 or 28, wherein said peptide is between six and one hundred amino acids in length.

30. The composition of Claims 19, 23 or 29, wherein said invasion-inducing agent is on a solid support.

31. The composition of Claim 30, wherein said solid support is a dressing.

32. A method for treating a wound, comprising:

a) providing: i) the invasion-inducing agent of Claims 19, 23, 29, 30 or 31, and ii) a subject having at least one wound; and

b) administering said invasion-inducing agent to said subject under conditions such that the healing of said wound is promoted.

33. The method of Claim 32, wherein said subject is a human.

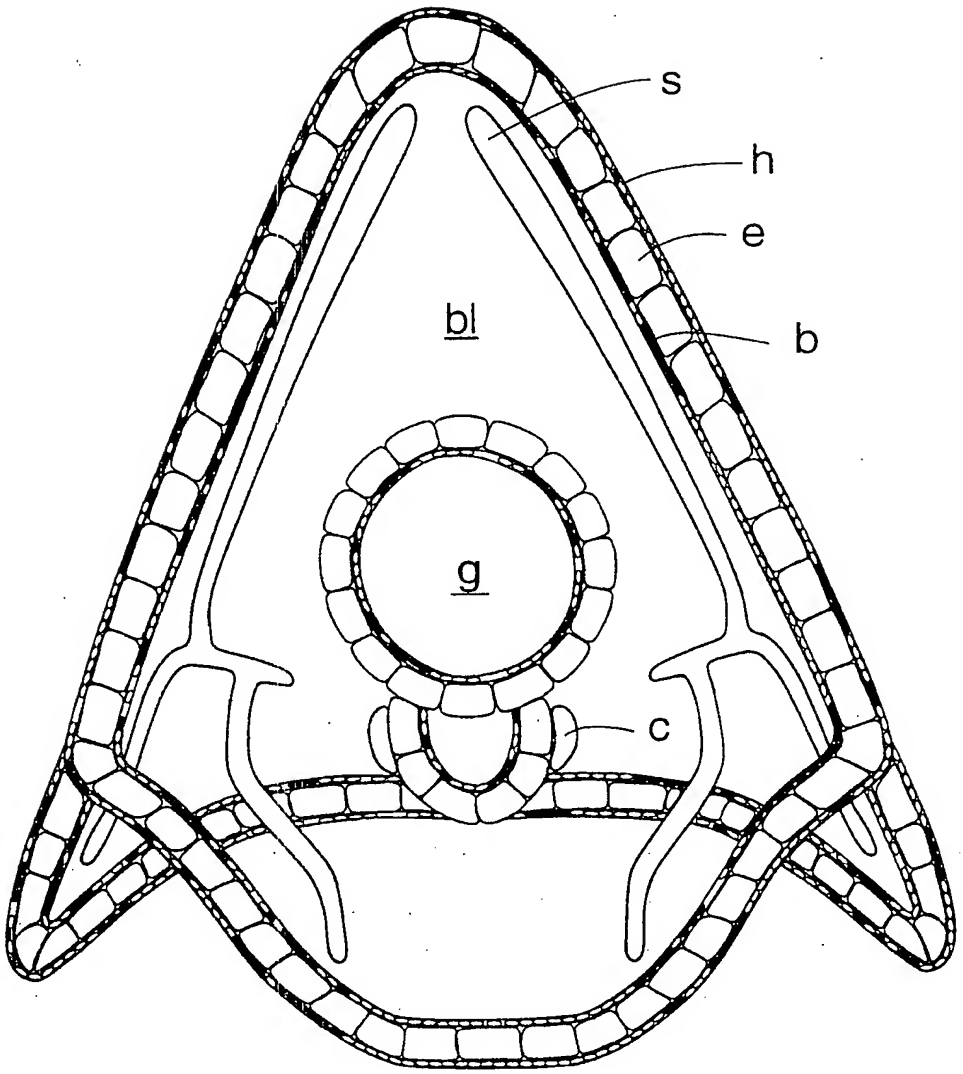


FIG. 1

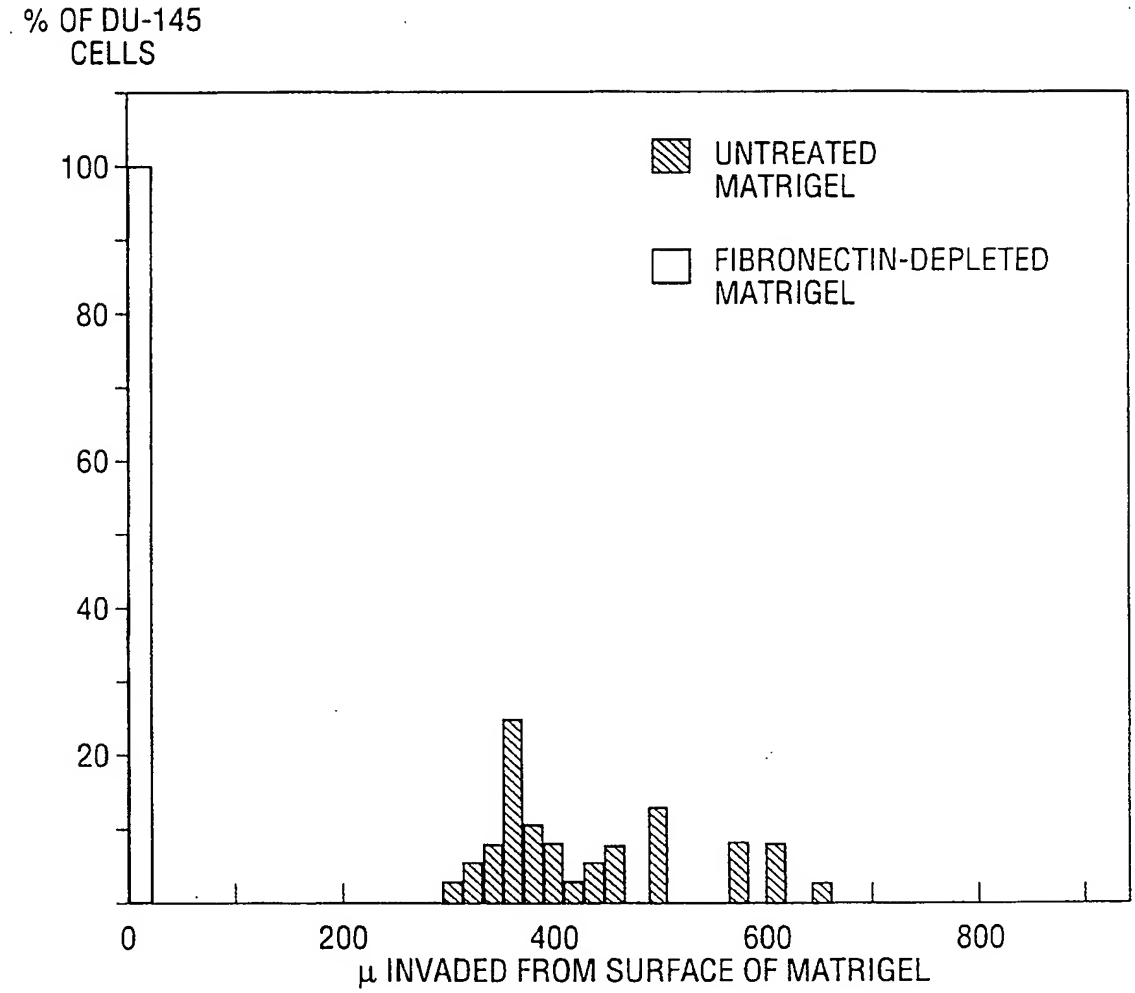

% OF DU-145 CELLS
100
80
60
40
20
0
200
400
600
800
μ INVADED FROM SURFACE OF MATRIGEL
UNTREATED MATRIGEL
FIBRONECTIN-DEPLETED MATRIGEL

FIG. 2

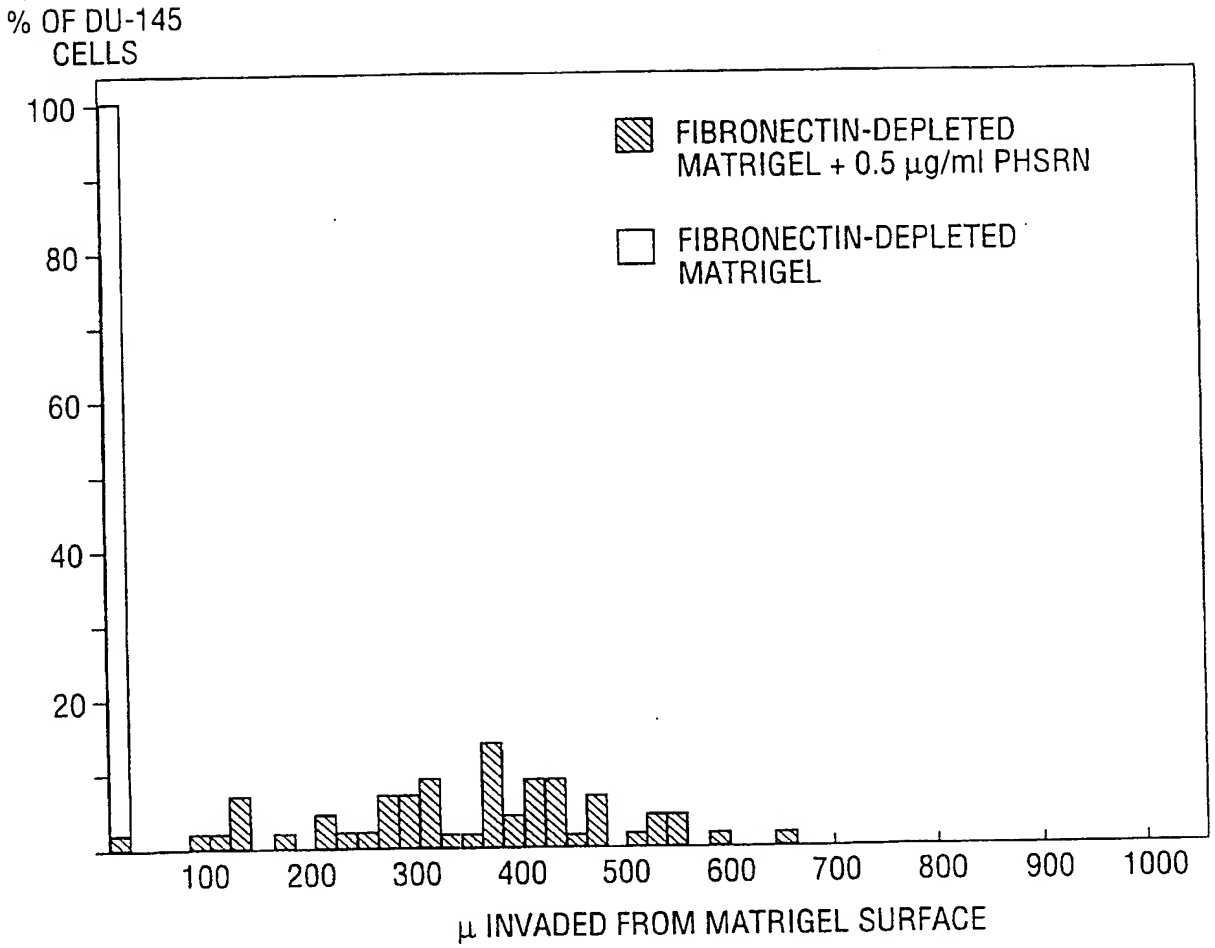

% OF DU-145 CELLS
100
80
60
40
20
FIBRONECTIN-DEPLETED MATRIGEL + 0.5 µg/ml PHSRN
FIBRONECTIN-DEPLETED MATRIGEL
100
200
300
400
500
600
700
800
900
1000
µ INVADED FROM MATRIGEL SURFACE

FIG. 3

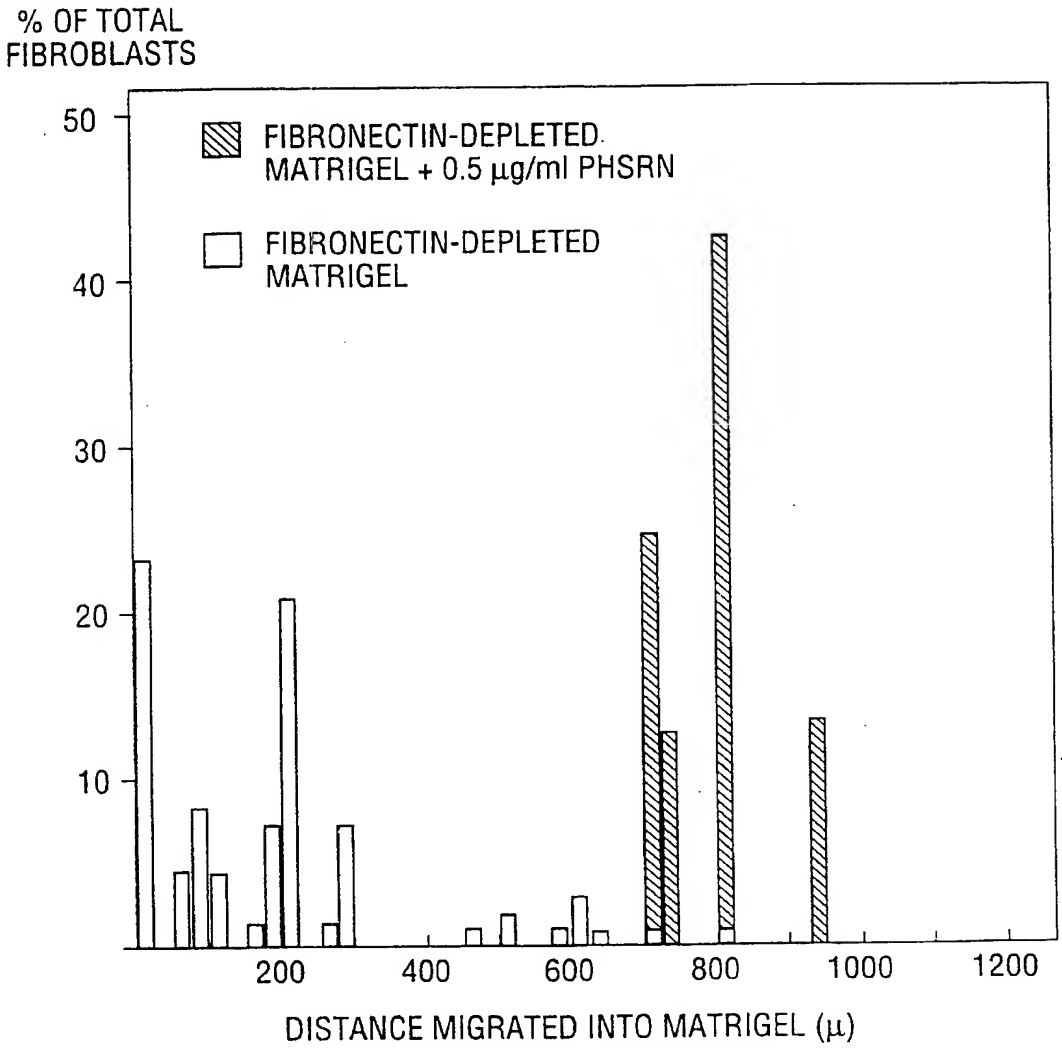

% OF TOTAL FIBROBLASTS
50
40
30
20
10
FIBRONECTIN-DEPLETED MATRIGEL + 0.5 µg/ml PHSRN
FIBRONECTIN-DEPLETED MATRIGEL
200
400
600
800
1000
1200
DISTANCE MIGRATED INTO MATRIGEL (µ)

FIG. 4

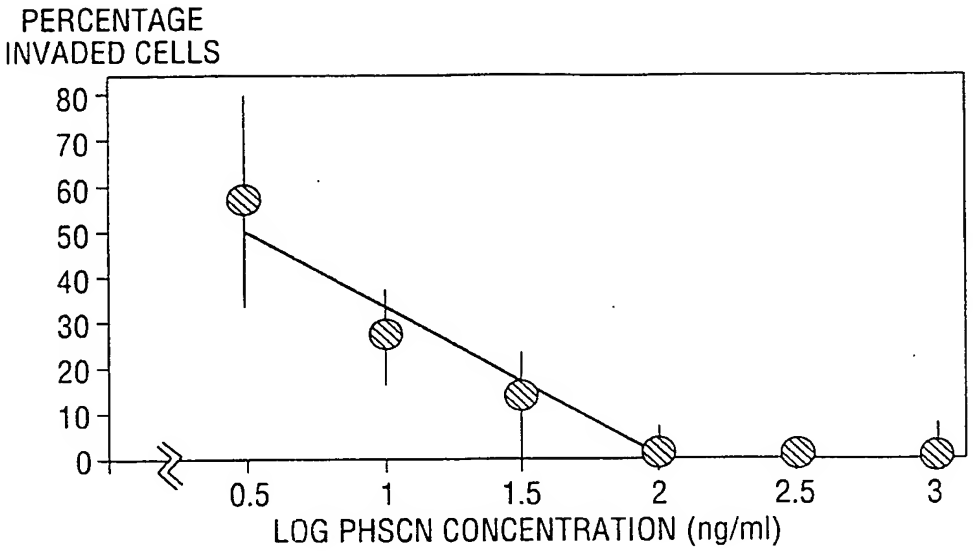

PERCENTAGE
INVADED CELLS
80
70
60
50
40
30
20
10
0
0.5
1
1.5
2
2.5
3
LOG PHSCN CONCENTRATION (ng/ml)

FIG. 5A

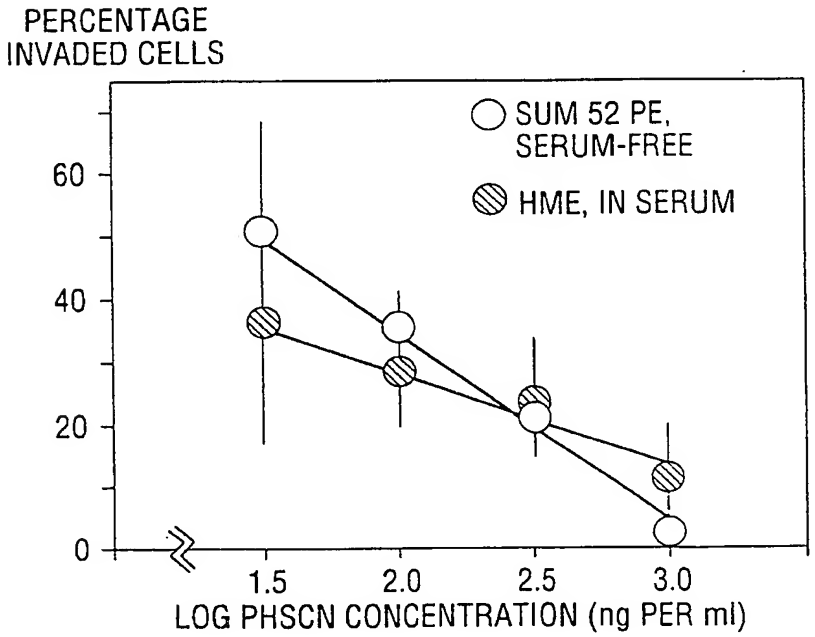

PERCENTAGE
INVADED CELLS
SUM 52 PE,
SERUM-FREE
HME, IN SERUM
60
40
20
0
1.5
2.0
2.5
3.0
LOG PHSCN CONCENTRATION (ng PER ml)

FIG. 5B

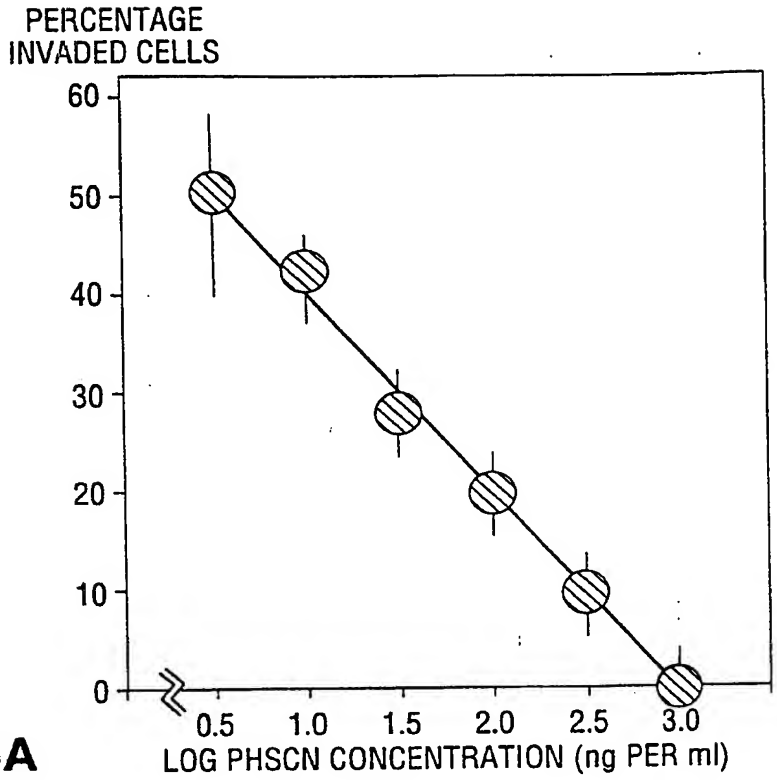

PERCENTAGE
INVADED CELLS
60
50
40
30
20
10
0
0.5
1.0
1.5
2.0
2.5
3.0
LOG PHSCN CONCENTRATION (ng PER ml)

FIG. 6A

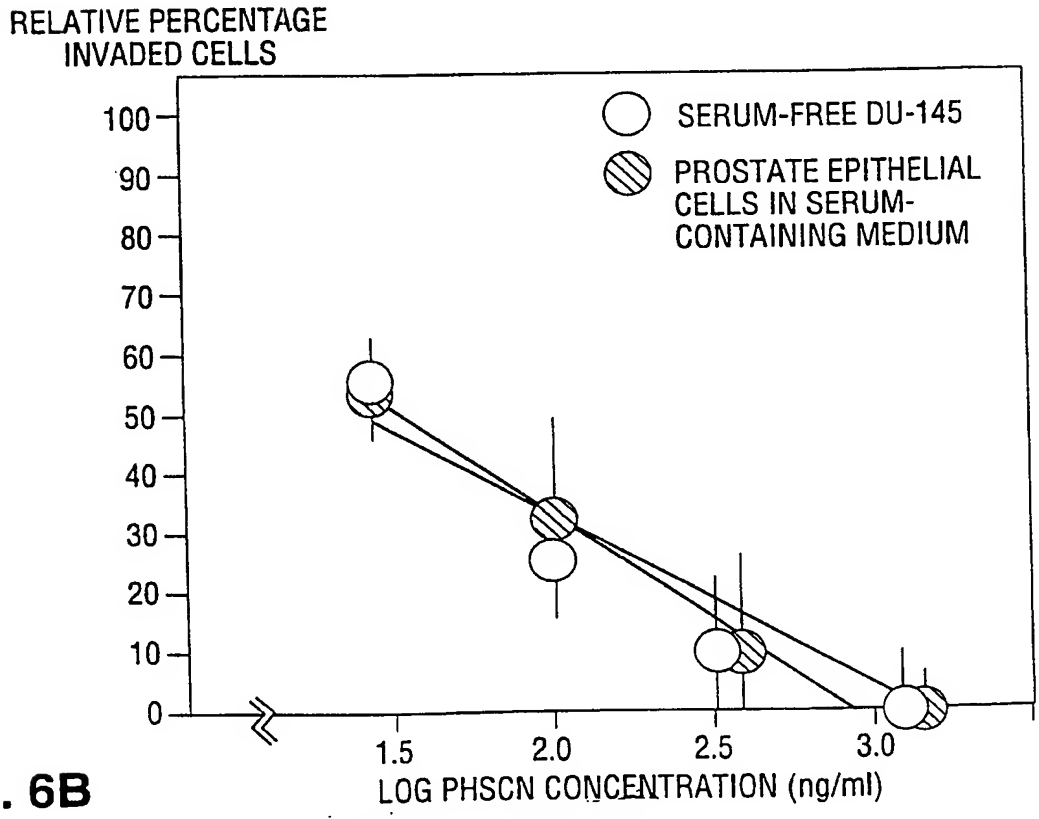

RELATIVE PERCENTAGE
INVADED CELLS
100
90
80
70
60
50
40
30
20
10
0
SERUM-FREE DU-145
PROSTATE EPITHELIAL CELLS IN SERUM-CONTAINING MEDIUM
1.5
2.0
2.5
3.0
LOG PHSCN CONCENTRATION (ng/ml)

FIG. 6B

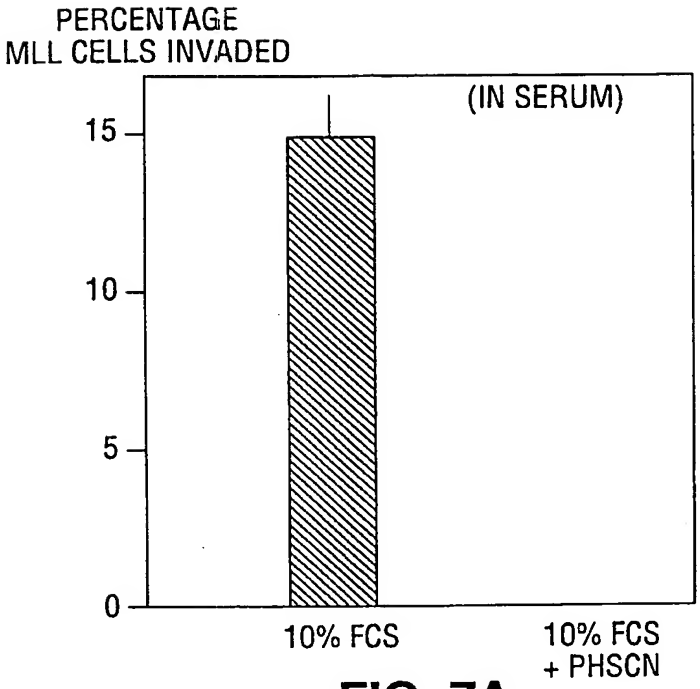

PERCENTAGE
MLL CELLS INVADED
(IN SERUM)
15
10
5
0
10% FCS
10% FCS
+ PHSCN

FIG. 7A

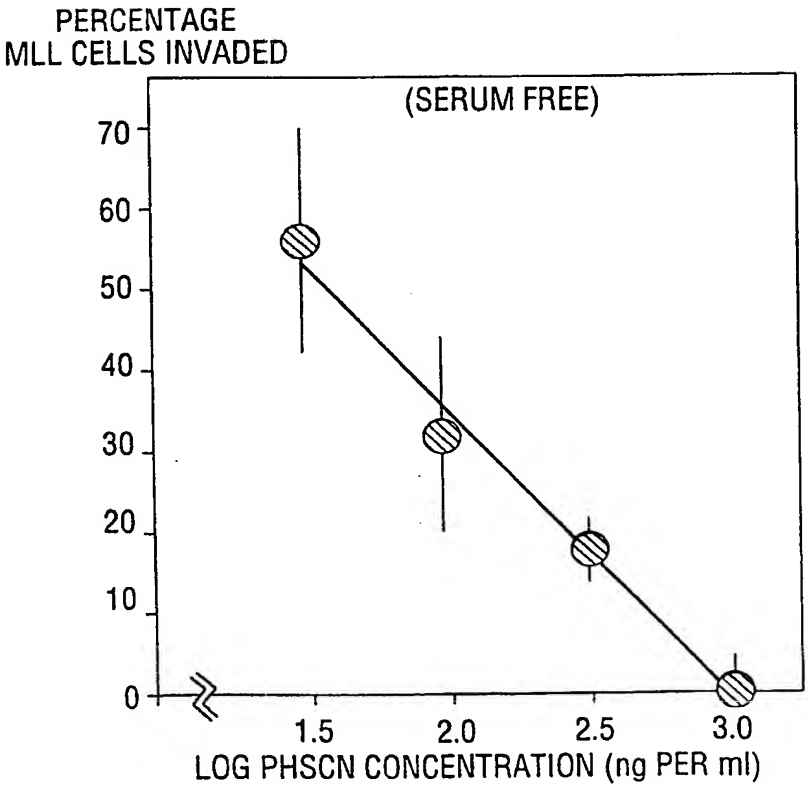

PERCENTAGE
MLL CELLS INVADED
(SERUM FREE)
70
60
50
40
30
20
10
0
1.5
2.0
2.5
3.0
LOG PHSCN CONCENTRATION (ng PER ml)

FIG. 7B

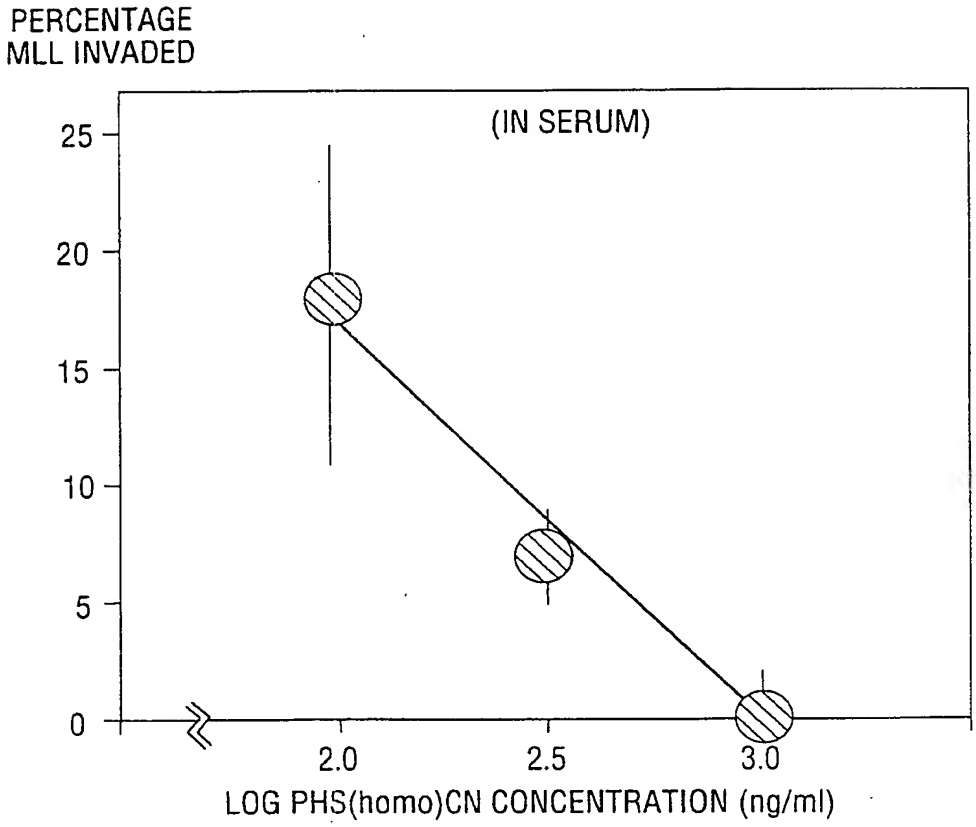

PERCENTAGE
MLL INVADED
(IN SERUM)
25
20
15
10
5
0
2.0
2.5
3.0
LOG PHS(homo)CN CONCENTRATION (ng/ml)

FIG. 8

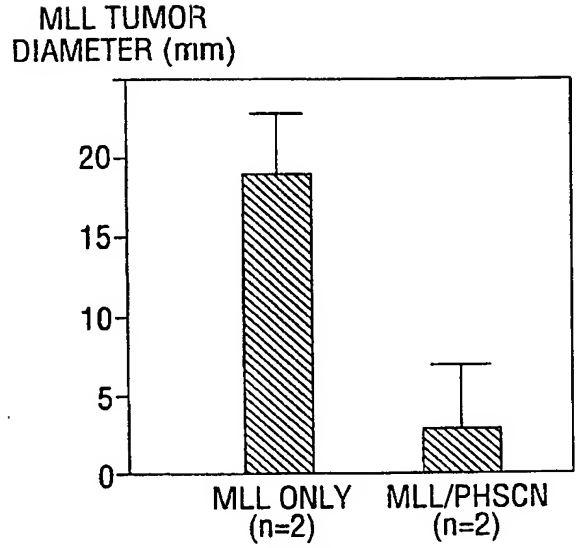

MLL TUMOR
DIAMETER (mm)
20
15
10
5
0
MLL ONLY
(n=2)
MLL/PHSCN
(n=2)

FIG. 9A

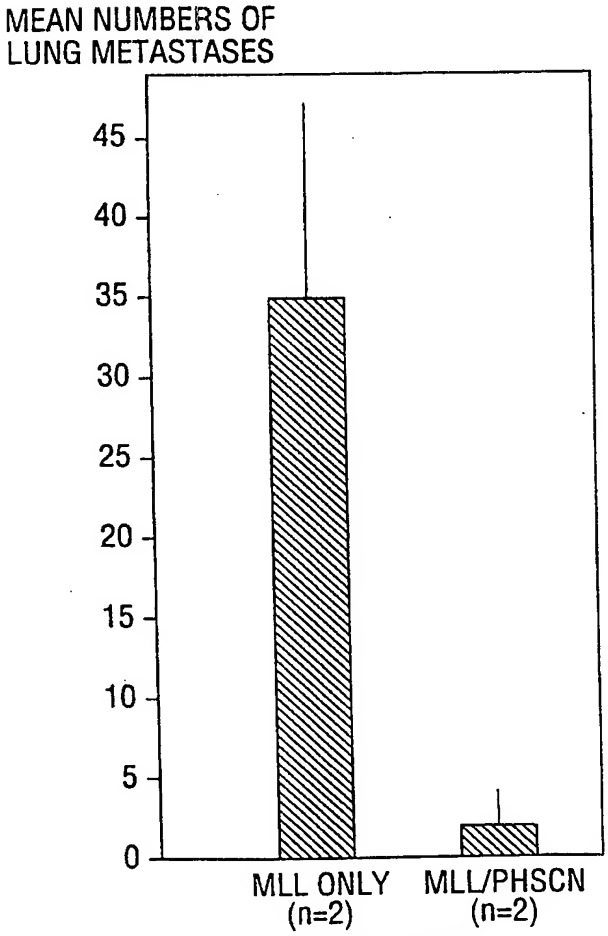

MEAN NUMBERS OF
LUNG METASTASES
45
40
35
30
25
20
15
10
5
0
MLL ONLY
(n=2)
MLL/PHSCN
(n=2)

FIG. 9B

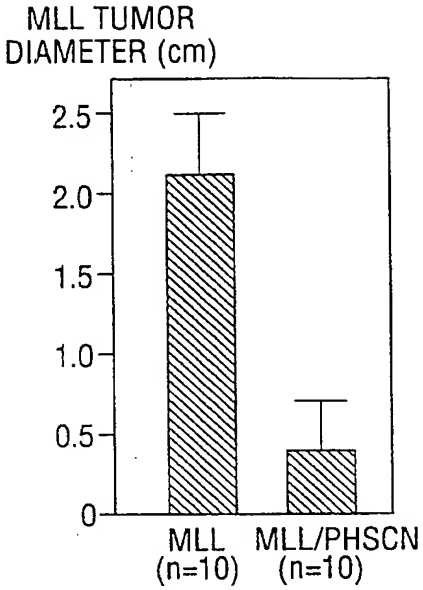


FIG. 10A

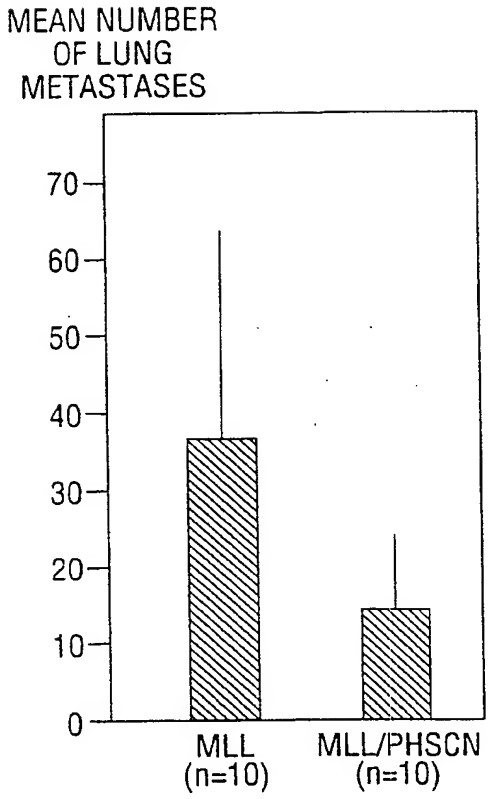


FIG. 10B

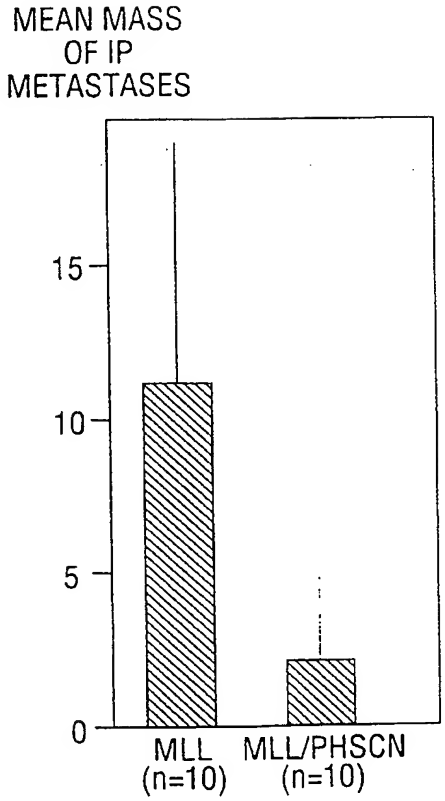


FIG. 10C

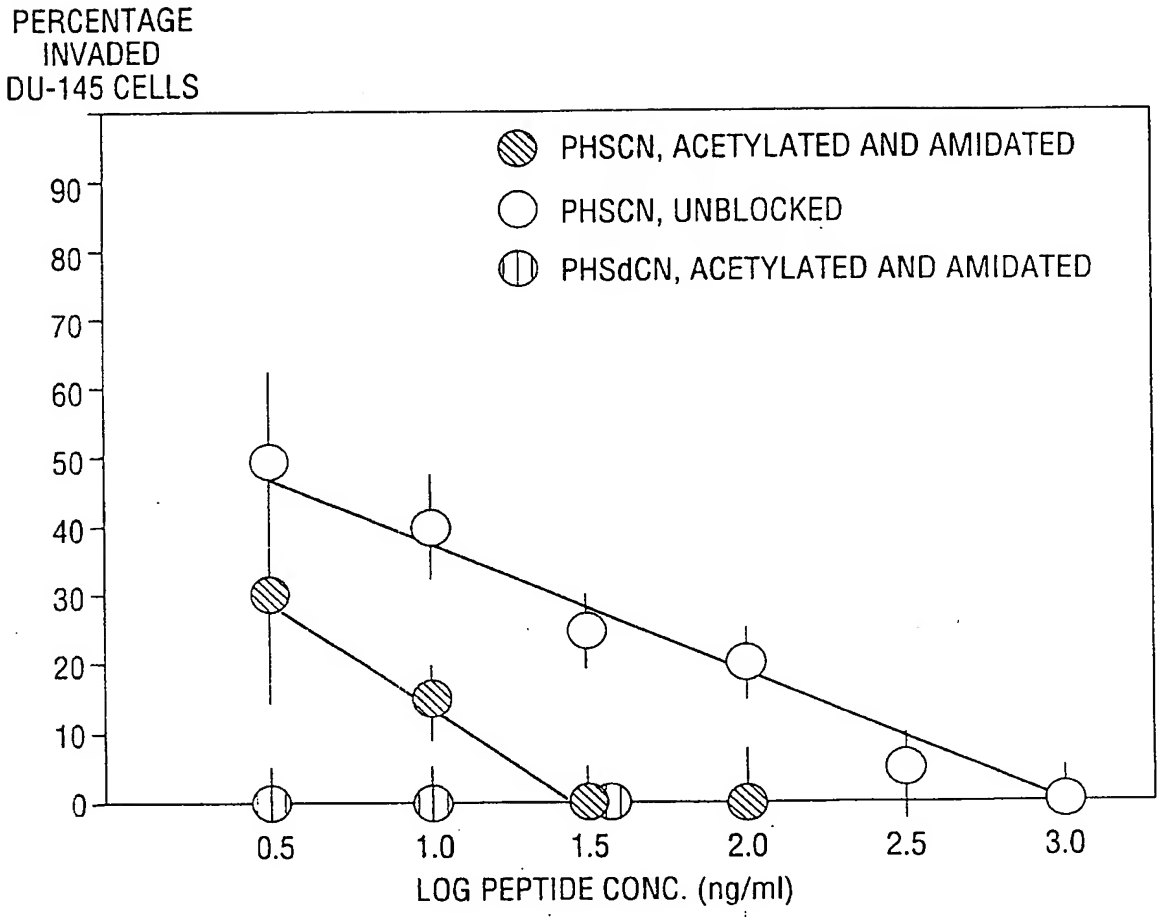

PERCENTAGE
INVADED
DU-145 CELLS
PHSCN, ACETYLATED AND AMIDATED
PHSCN, UNBLOCKED
PHSdCN, ACETYLATED AND AMIDATED
90
80
70
60
50
40
30
20
10
0
0.5
1.0
1.5
2.0
2.5
3.0
LOG PEPTIDE CONC. (ng/ml)

FIG. 11

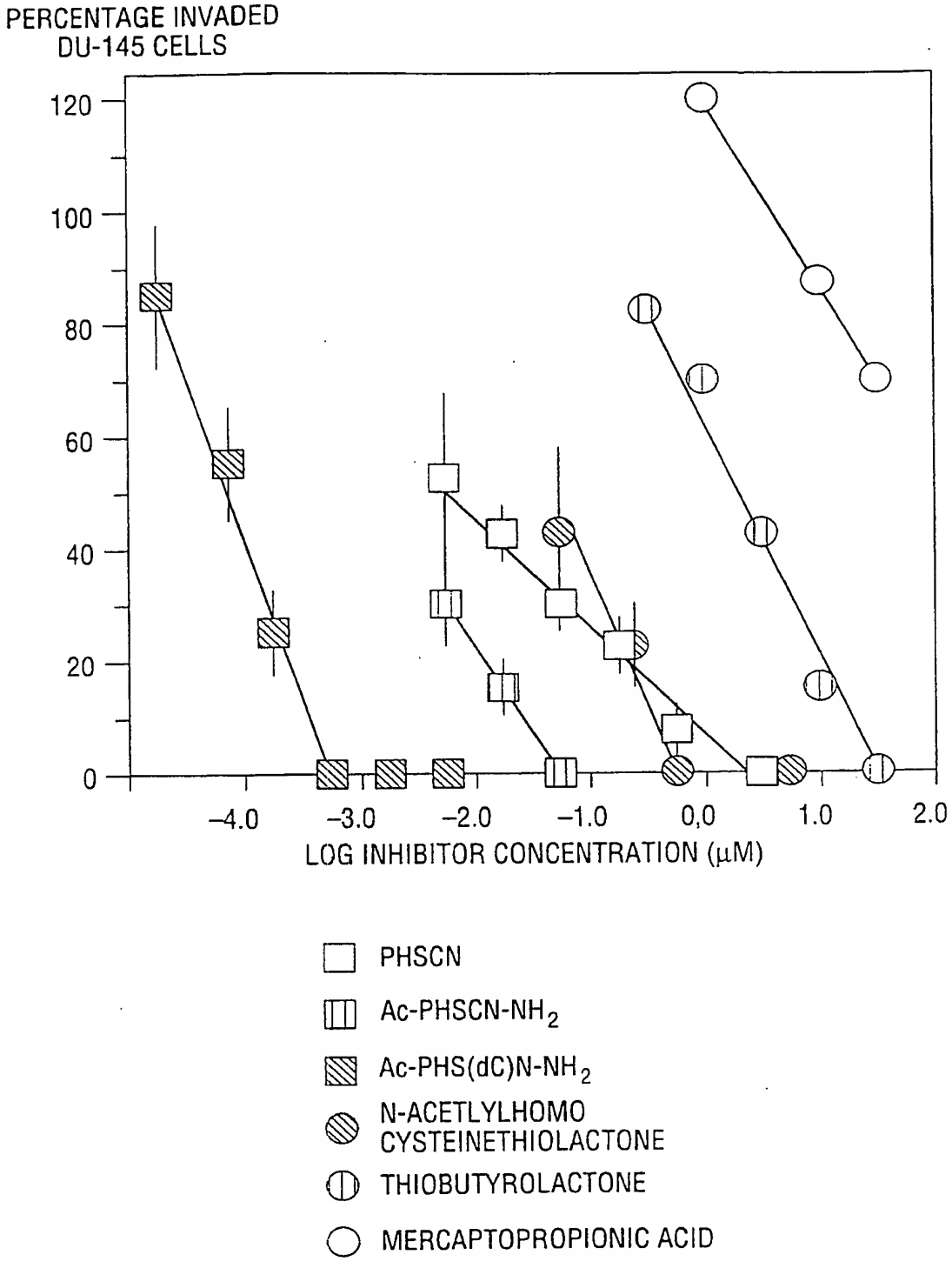
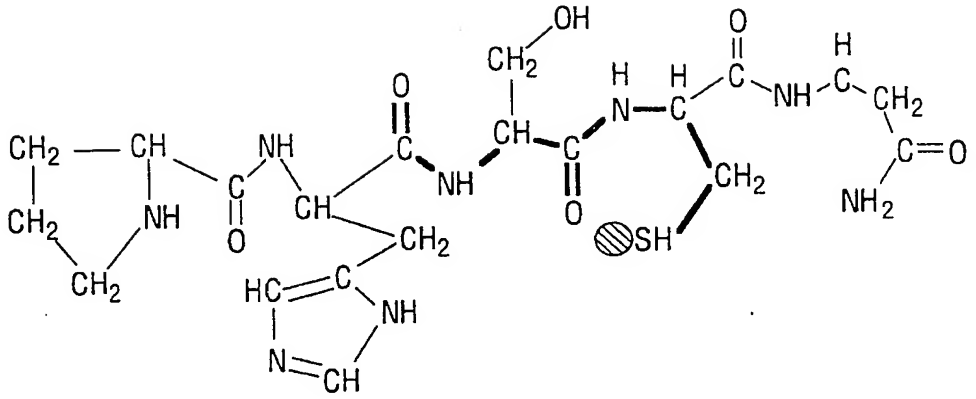
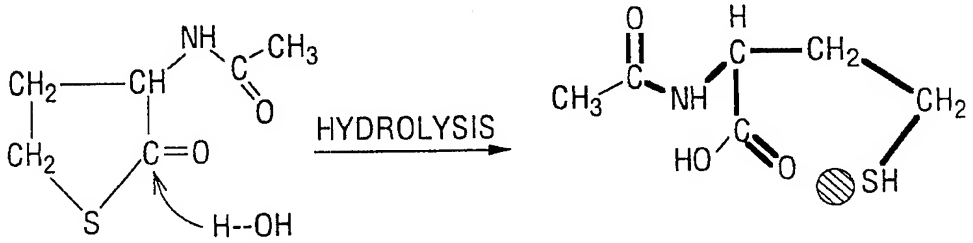

PERCENTAGE INVADED
DU-145 CELLS
120
100
80
60
40
20
0
-4.0
-3.0
-2.0
-1.0
0,0
1.0
2.0
LOG INHIBITOR CONCENTRATION (μM)
PHSCN
Ac-PHSCN-NH2
Ac-PHS(dC)N-NH2
N-ACETLYLHOMO CYSTEINETHIOLACTONE
THIOBUTYROLACTONE
MERCAPTOPROPIONIC ACID

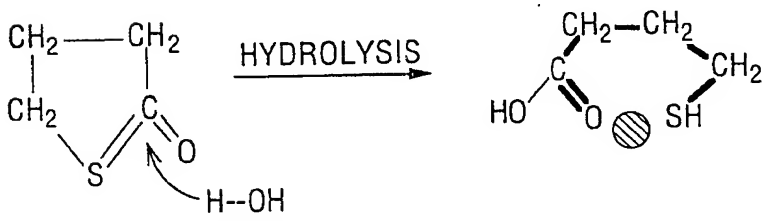
FIG. 12



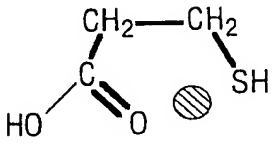
PROLINE--HISTIDINE--SERINE--CYSTEINE--ASPARAGINE



N-ACETYLHOMOCYSTEINETHIOLACTONE



THIOBUTYROLACTONE



MERCAPTOPROPIONIC ACID

⊗ — DIVALENT CATION

FIG. 13

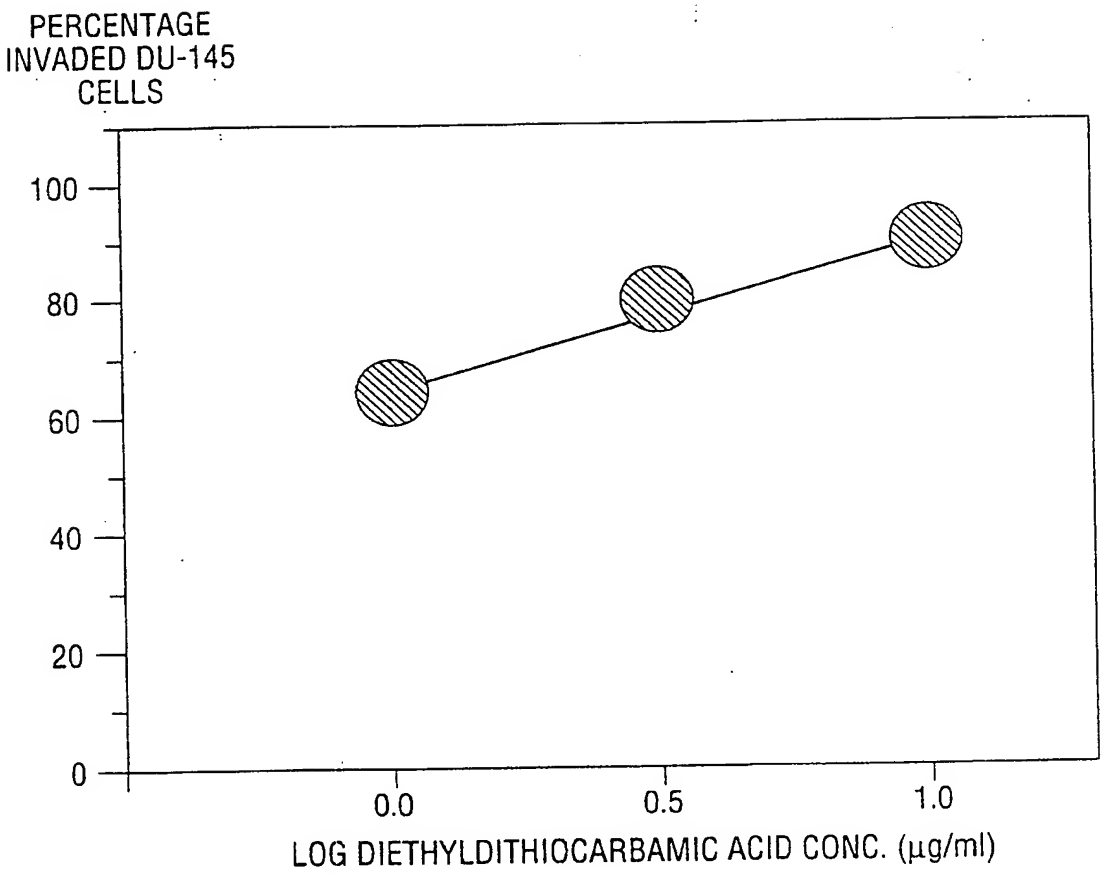

PERCENTAGE
INVADED DU-145
CELLS
100
80
60
40
20
0
0.0
0.5
1.0
LOG DIETHYLDITHIOCARBAMIC ACID CONC. (µg/ml)

FIG. 14

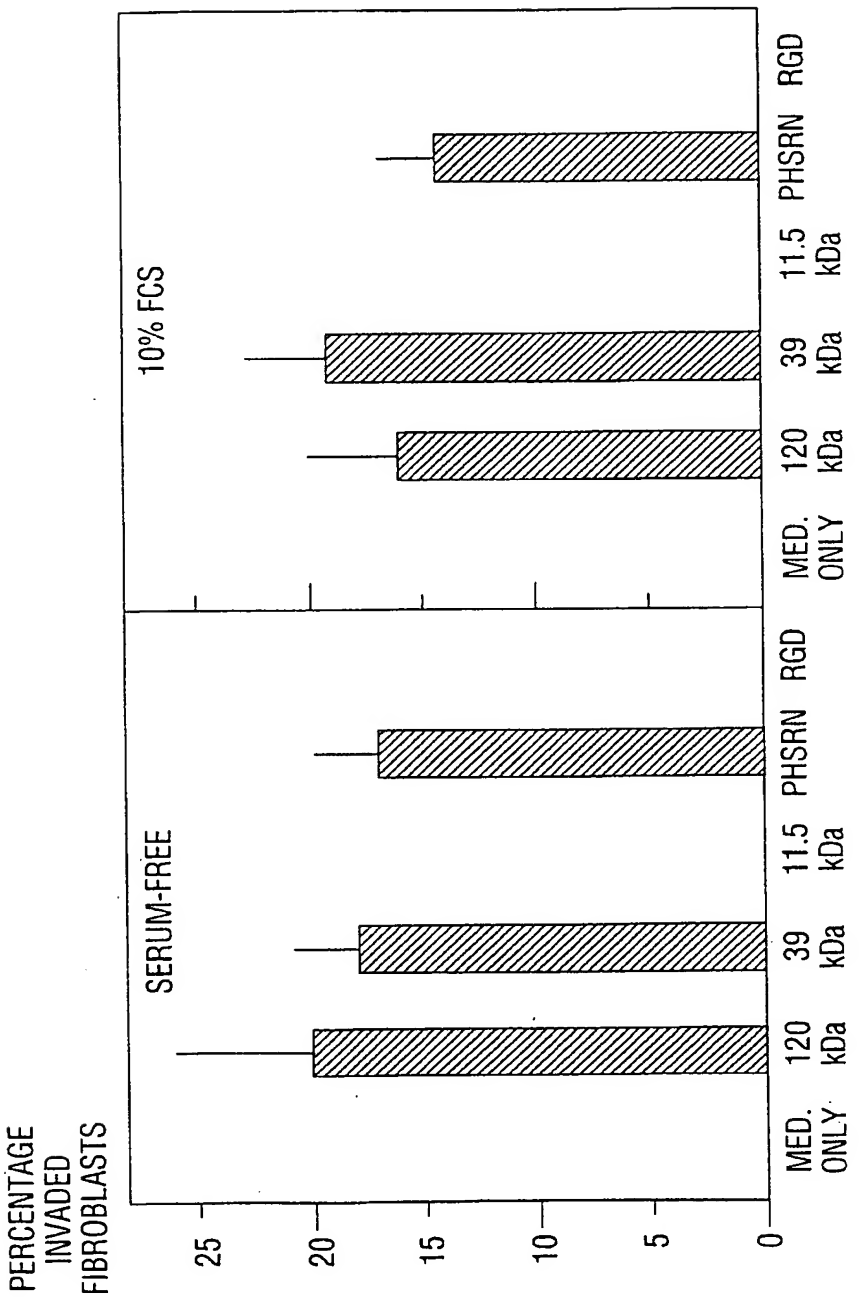

PERCENTAGE INVADED FIBROBLASTS
0
5
10
15
20
25
SERUM-FREE
10% FCS
MED. ONLY
120 kDa
39 kDa
11.5 kDa
PHSRN
RGD

FIG. 15

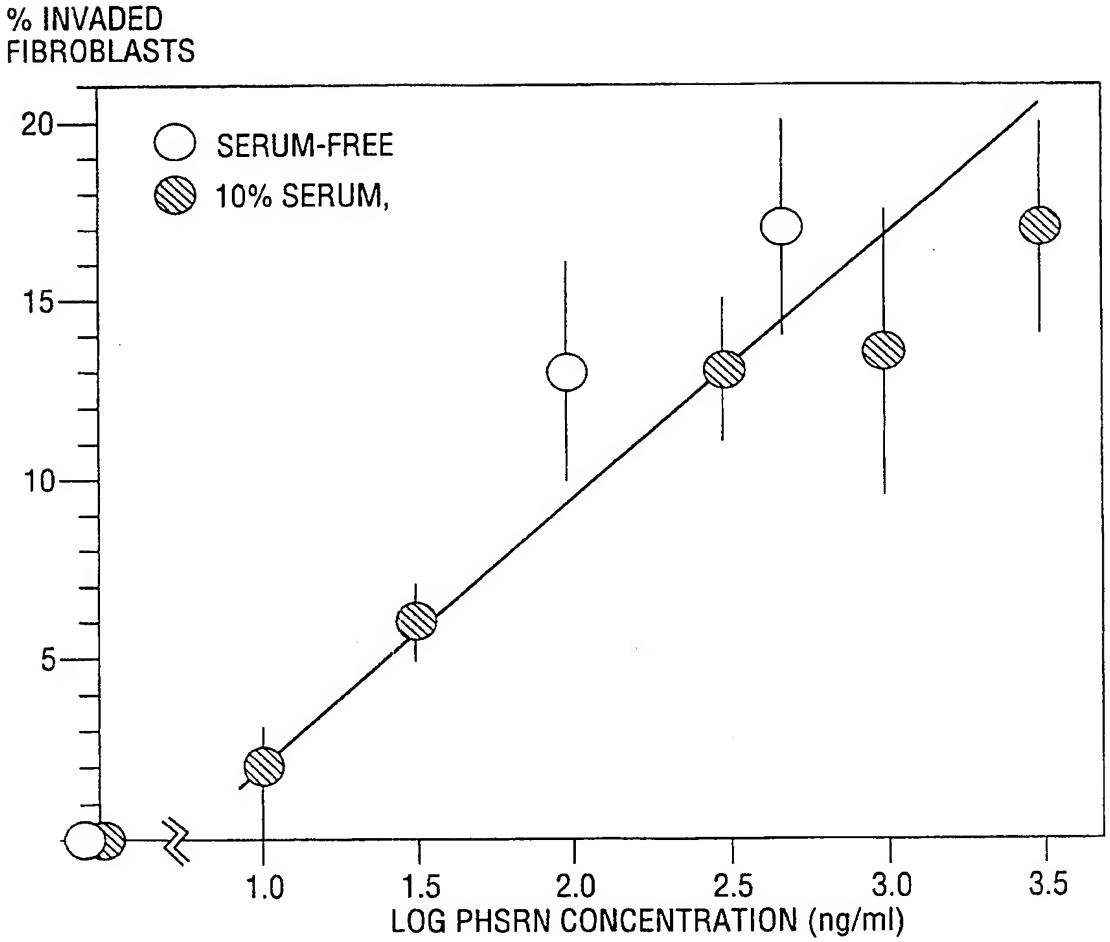

% INVADED
FIBROBLASTS
20
15
10
5
SERUM-FREE
10% SERUM,
1.0
1.5
2.0
2.5
3.0
3.5
LOG PHSRN CONCENTRATION (ng/ml)

FIG. 16

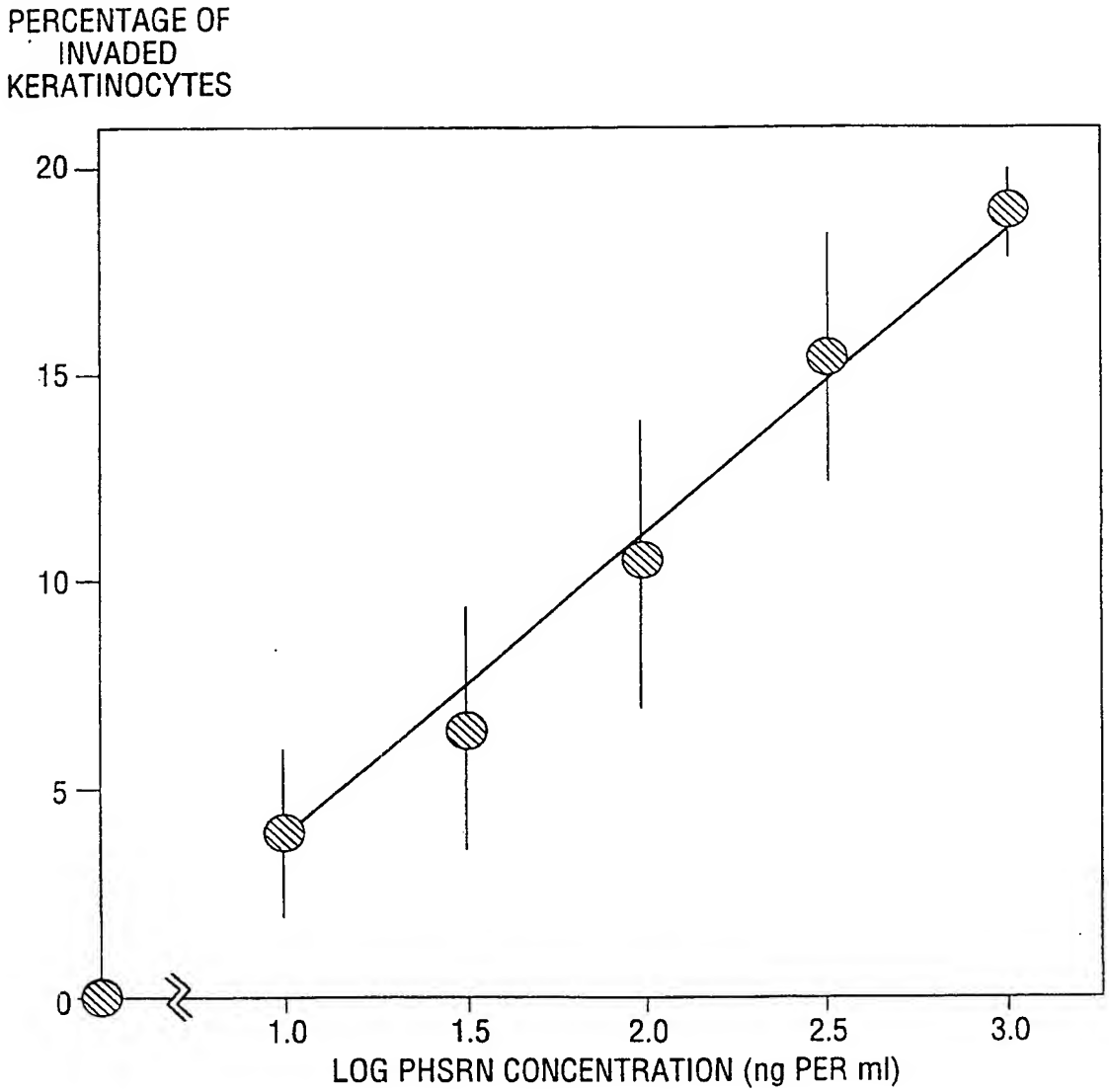

PERCENTAGE OF
INVADED
KERATINOCYTES
20
15
10
5
0
1.0
1.5
2.0
2.5
3.0
LOG PHSRN CONCENTRATION (ng PER ml)

FIG. 17

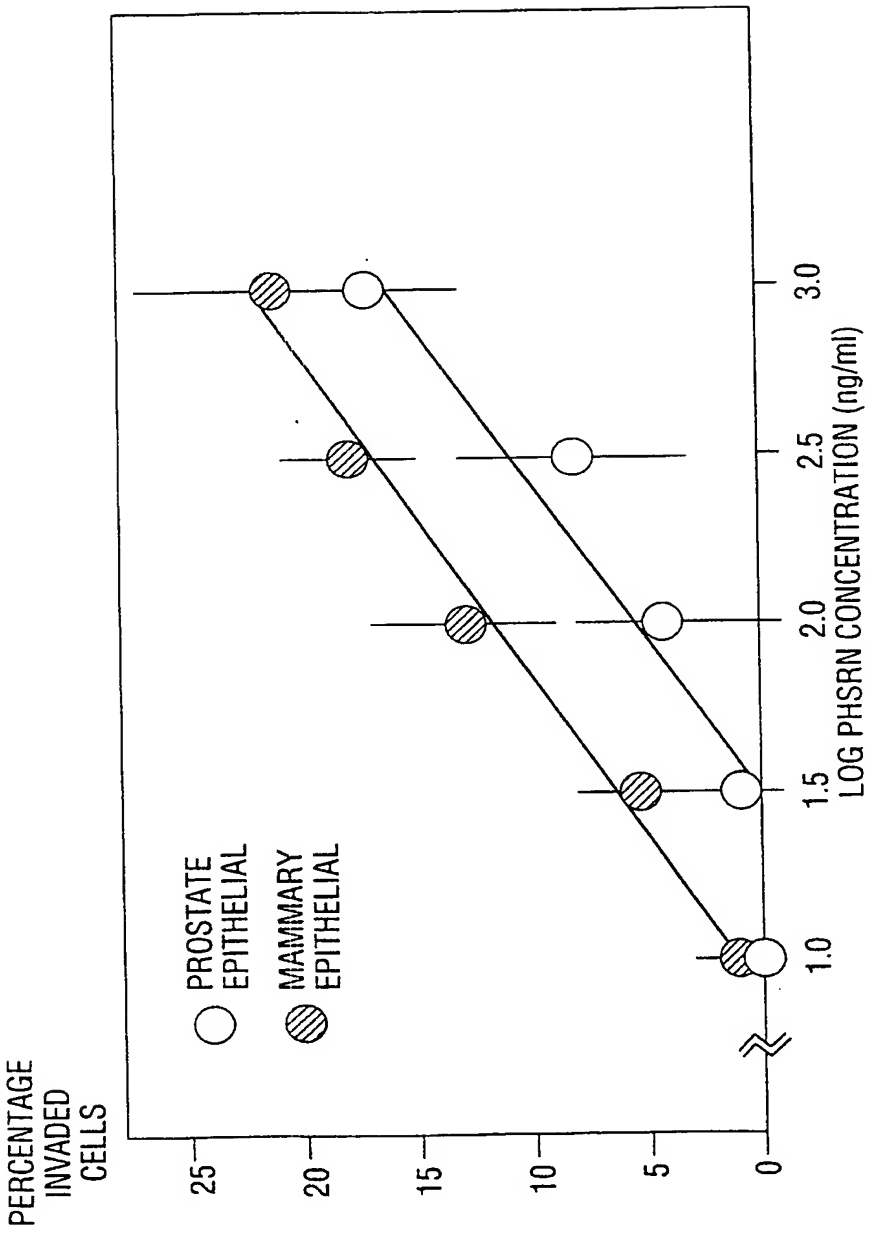

PERCENTAGE INVADED CELLS
25
20
15
10
5
0
PROSTATE EPITHELIAL
MAMMARY EPITHELIAL
1.0
1.5
2.0
2.5
3.0
LOG PHSRN CONCENTRATION (ng/ml)

FIG. 18

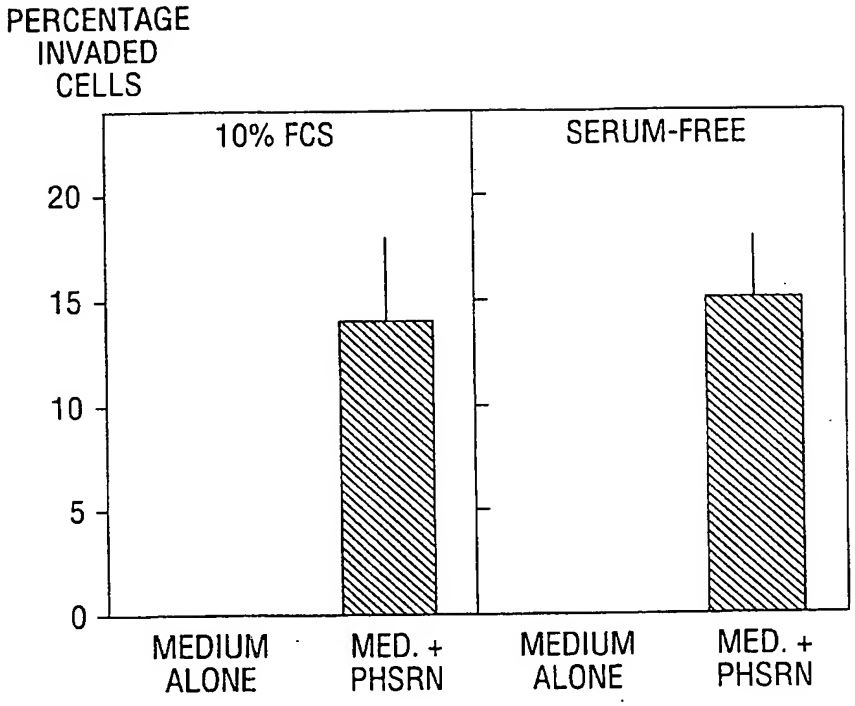

PERCENTAGE
INVADED
CELLS
10% FCS
SERUM-FREE
20
15
10
5
0
MEDIUM
ALONE
MED. +
PHSRN
MEDIUM
ALONE
MED. +
PHSRN

FIG. 19

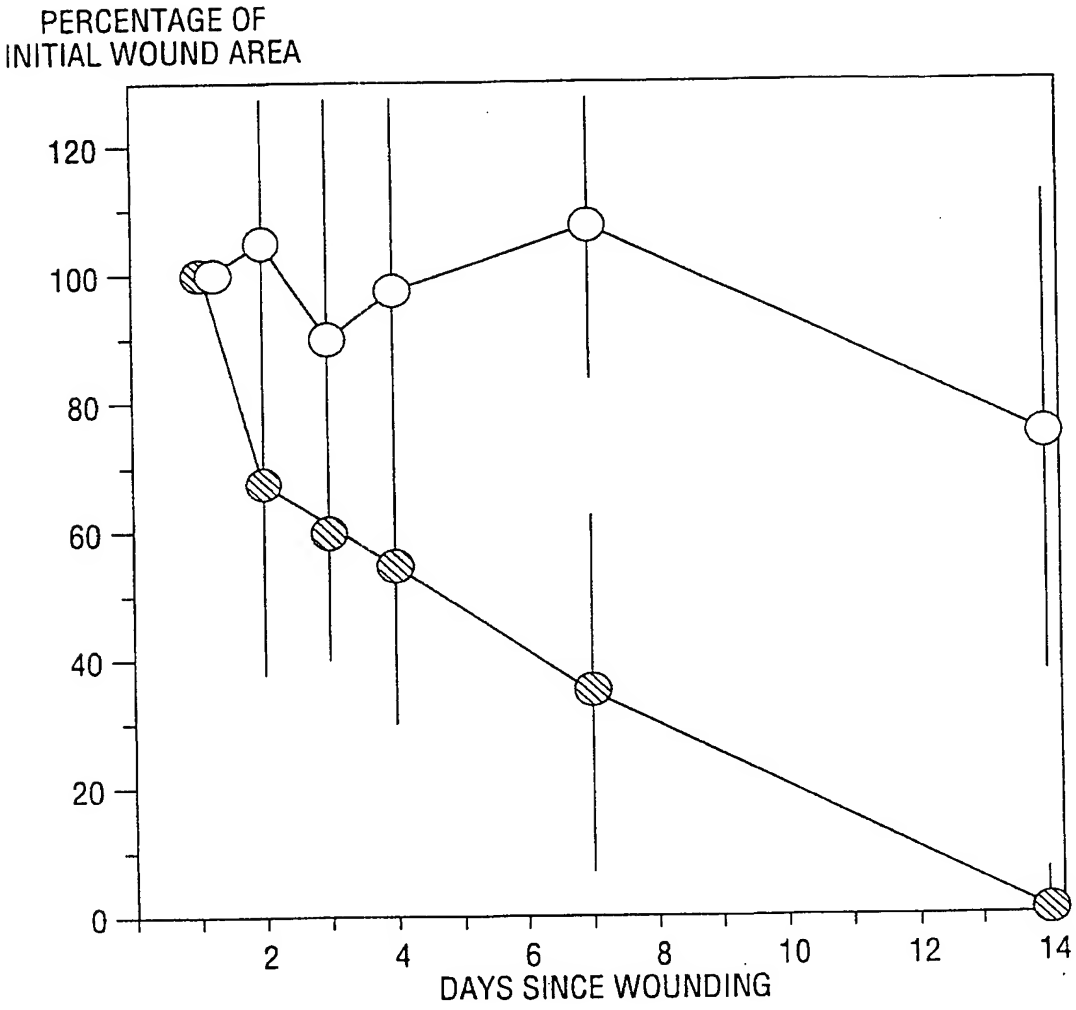

PERCENTAGE OF
INITIAL WOUND AREA
120
100
80
60
40
20
0
2
4
6
8
10
12
14
DAYS SINCE WOUNDING
UNTREATED C57B16Ks db/db
PHSRN-TREATED C57B16Ks db/db

FIG. 20

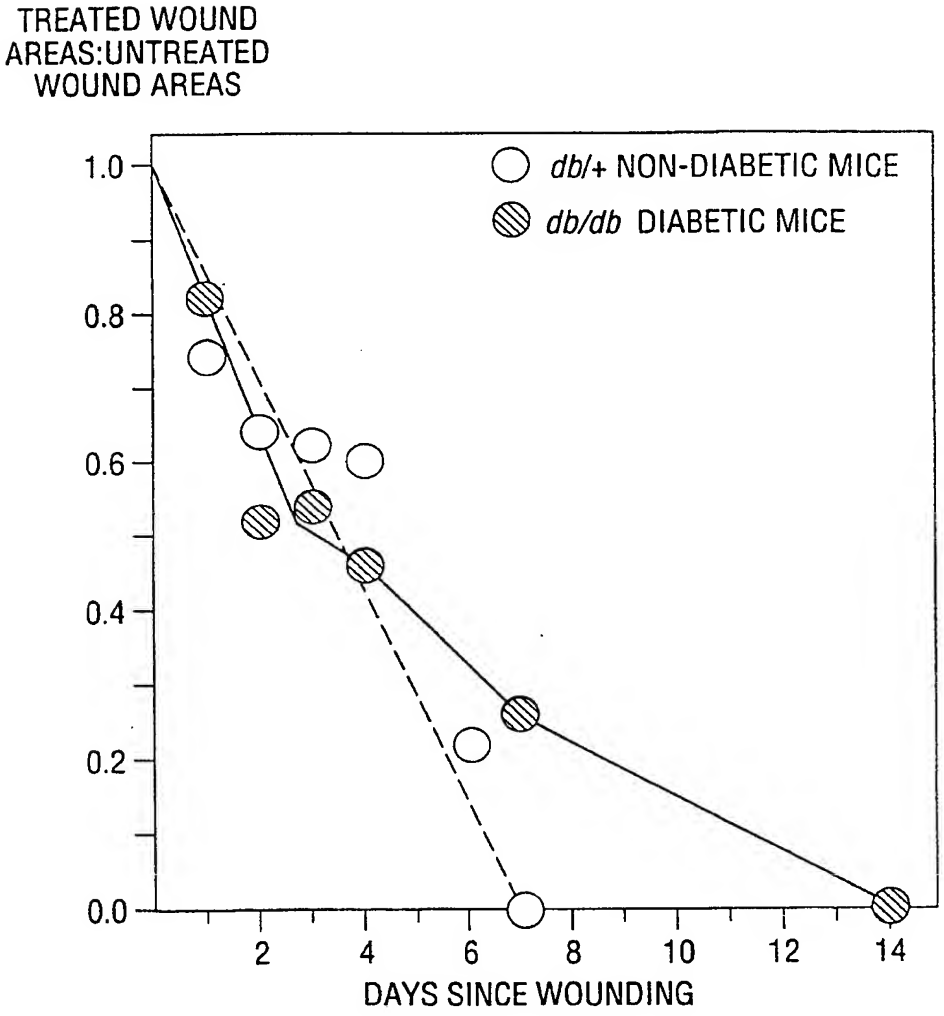

TREATED WOUND AREAS:UNTREATED WOUND AREAS
1.0
0.8
0.6
0.4
0.2
0.0
db/+ NON-DIABETIC MICE
db/db DIABETIC MICE
2
4
6
8
10
12
14
DAYS SINCE WOUNDING

FIG. 21

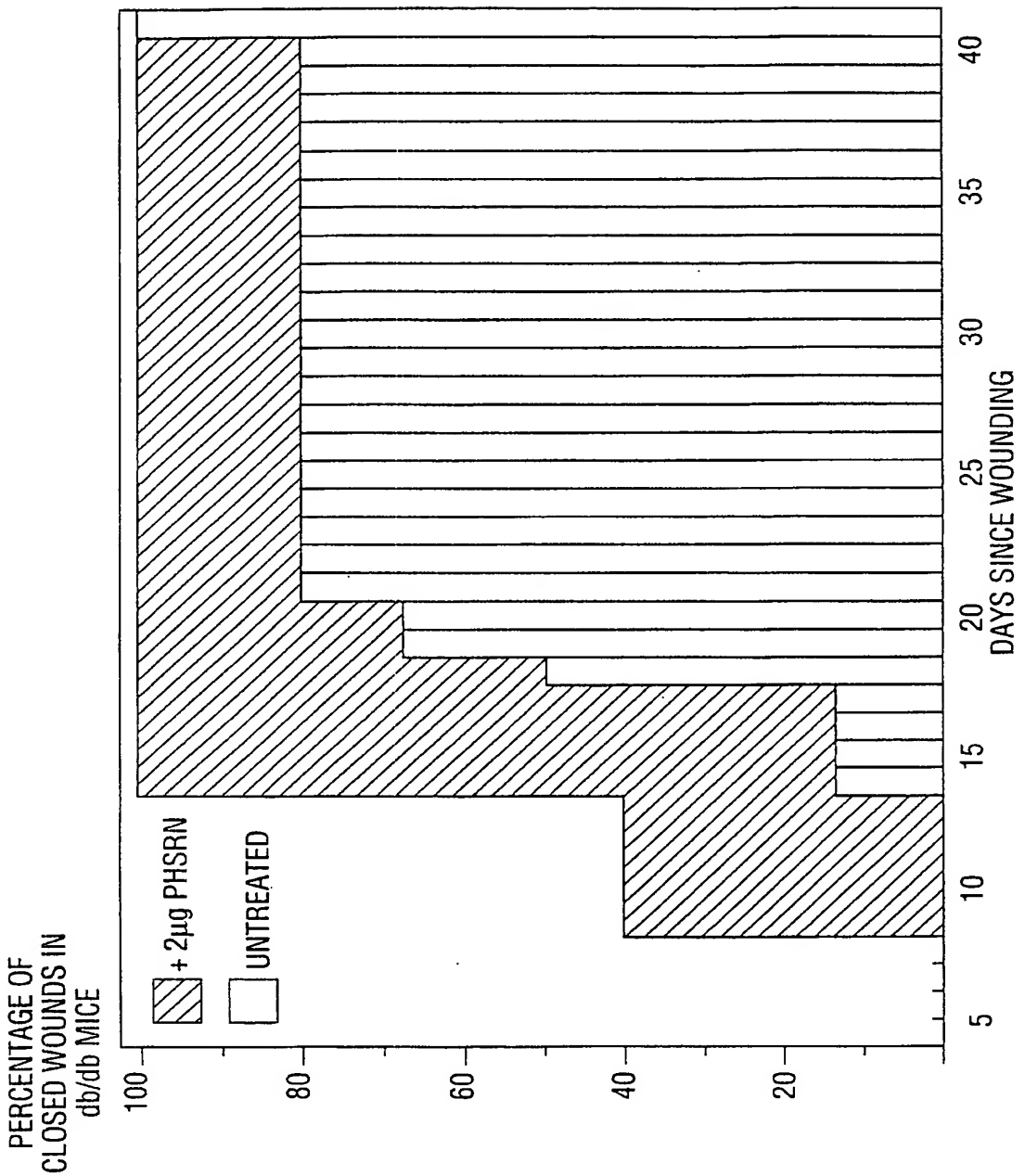

PERCENTAGE OF CLOSED WOUNDS IN db/db MICE
100
80
60
40
20
+ 2µg PHSRN
UNTREATED
5
10
15
20
25
30
35
40
DAYS SINCE WOUNDING

FIG. 22

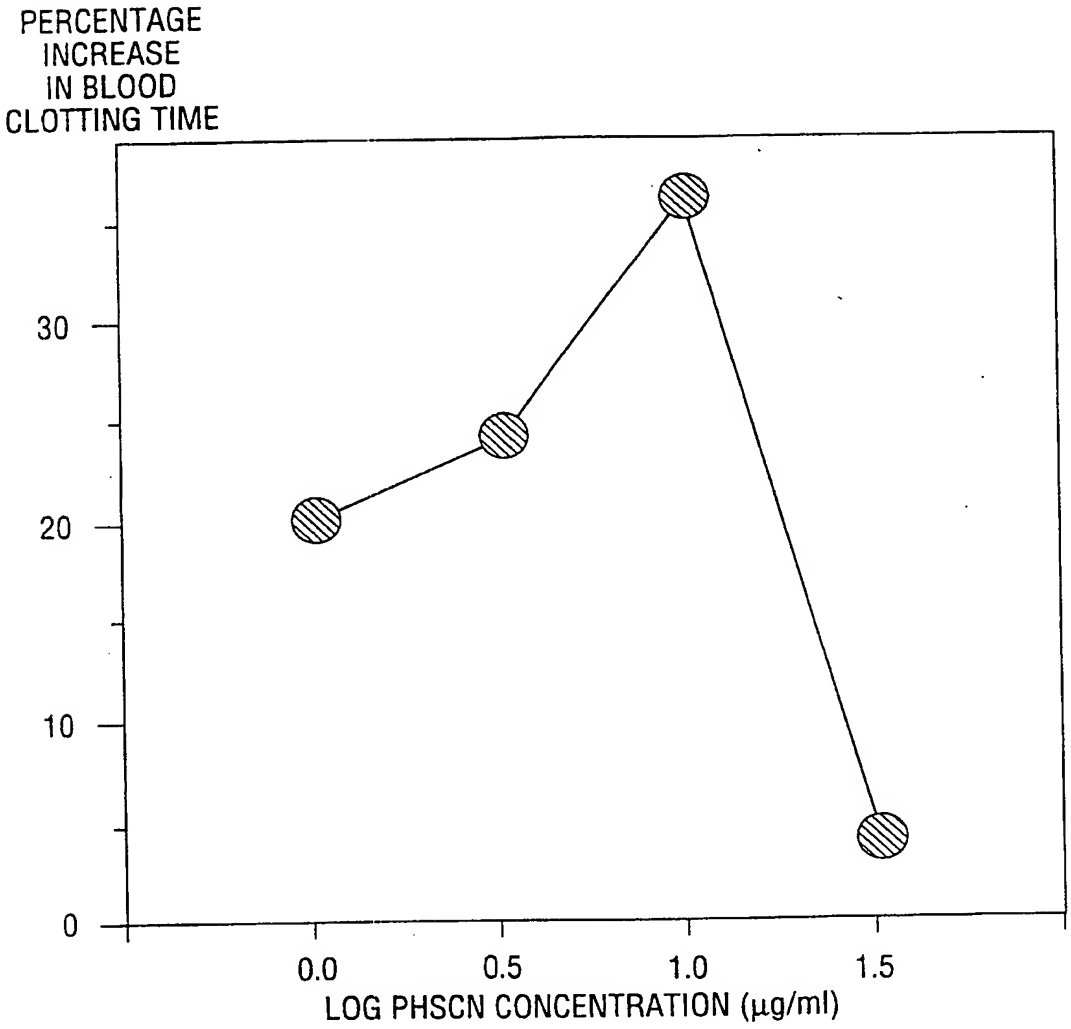

PERCENTAGE
INCREASE
IN BLOOD
CLOTTING TIME
30
20
10
0
0.0
0.5
1.0
1.5
LOG PHSCN CONCENTRATION (µg/ml)

FIG. 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/21674

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/29, 4, 7.21, 7.23, 7.1, 69.1; 530/300, 330, 382; 514/802; 2, 17

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS: PHSRN
APS: PHSRN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MORI, H. et al. Preparation of cell adhesion protein-like peptides as cancer metastasis inhibitor. JP 06298797 A2, 25 October 1994, see the english abstract.	1-2, 4-6, 14, 19 12-20, 22-26, 29-30
Y	AKIYAMA, S. et al. Fibronectin and integrins in invasion and metastasis. Cancer and Metastasis Review. 1995, Vol. 14, No. 3, see the abstract.	1-2, 4-6, 14, 19, 12-20, 22-26, 29-30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

23 JANUARY 1998

Date of mailing of the international search report

23 FEB 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer

LOUISE LEARY

Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/21674

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12Q 1/02, 1/00,; G01N 33/567, 33/574, 33/53; C12P 21/06; A61K 38/00, 38/04, 35/14; A01N 37/18

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/29, 4, 7.21, 7.23, 7.1, 69.1; 530/300, 330, 382; 514/802; 2, 17